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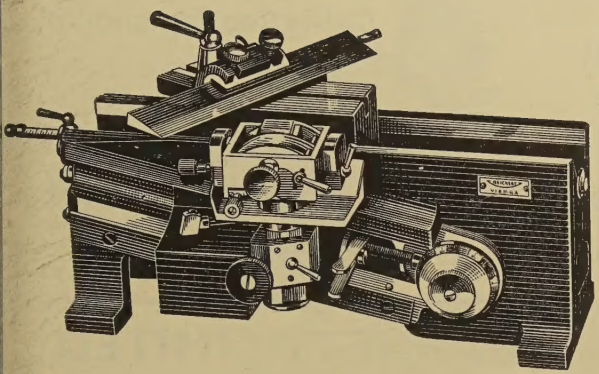

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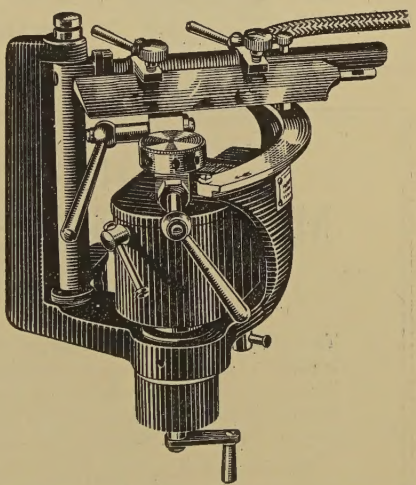

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[1 *front*]

The Histochemical Localization of β-Glucuronidase in the Liver of the Newt

By F. BILLETT AND S. M. McGEE-RUSSELL

From the Department of Zoology and Comparative Anatomy, Oxford. Present addresses: F. Billett, Institute of Animal Genetics, West Mains Road, Edinburgh 9; S. M. McGee-Russell, Zoology Dept., Birkbeck College, Malet St., London, W.C. 1)

With one plate (fig. 1)

Summary

The localization of β-glucuronidase in the newt liver, by the use of quinolyl-8-glucuronide as a substrate, is described. The technique previously used for the digestive gland of the Roman snail may be applied successfully to the newt tissue. Results suggest, however, that the treatments involved produce considerable destruction of the fine structure of the cytoplasm, and that present techniques based upon the precipitation of the ferric salt of 8-hydroxyquinoline cannot give a fine intracellular localization of the enzyme.

Introduction

In the previous paper it was shown that β-glucuronidase could be localized satisfactorily in the digestive gland of *Helix pomatia* by the precipitate of ferric 8-hydroxyquinoline which forms in sections when incubated in a mixture containing quinolyl-8-glucuronide and ferric ions (Billett and McGee-Russell, 1955). The crystals which form are sufficiently characteristic to localize the enzyme, and conversion of them into Prussian blue (Friedenwald and Becker, 1948) appears to be unnecessary, and possibly misleading.

During application of the method to various vertebrate tissues it was discovered that newt liver gave a strong reaction for β-glucuronidase. This reaction was comparable in intensity to that given by the digestive gland of *Helix pomatia* and considerably stronger than that given by mouse kidney, spleen, and liver. β-Glucuronidase does not appear to have been described as present in newt liver before.

The predominant cells of the newt liver are large, and filled with numerous filamentous bodies, which are stained readily by the Altmann acid fuchsin technique for mitochondria. One of the objects of the work described here was to determine the effect of some of the reagents used in the histochemical technique upon these intracellular bodies, which are, presumably, mitochondria.

Materials

Triturus vulgaris and *T. cristatus* were obtained from ponds near Oxford. All reagents were prepared as described in the previous paper.

Method

The newts were killed by decapitation. The liver was removed immediately, washed in ice-cold saline, and transferred without delay into ice-cold formal-

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dehyde-saline. After fixation for 3–4 hours and a rinse in distilled water, the tissue was sectioned on a freezing microtome, usually at 10 μ, but in some cases at 6 μ or 8 μ. The sections were treated exactly as described in the previous paper. The substrate was prepared at various levels of pH between 4·0 and 5·0. A substrate pH in the region of 4·0 appeared to be optimal for the formation of the ferric 8-hydroxyquinoline crystals. Nuclei were counterstained in Mayer's haemalum for 3–5 minutes.

Results

Under optimal conditions, crystals of ferric 8-hydroxyquinoline were deposited in the sections within 1–4 hours. The deposits were usually very heavy, making the sections appear black to the unaided eye. Control sections, in which the enzyme was inhibited by 0·0005 M potassium hydrogen saccharate, did not contain any crystalline deposits. The crystals formed in the test sections were small, rarely exceeding 2 μ in length; they were often irregular; many took the form of short rods, rounded at each end.

The crystals appeared to be confined to the predominant cell type of the liver; they were almost completely absent from the characteristic layer of lymphoid cells which surrounds this organ in the newt. The crystals were confined to the cytoplasm of the liver cells (fig. 1, A and B).

Failure of saccharate controls at low pH

During the course of the investigation it was observed that with substrates of low pH, below 4·0, the saccharate failed to inhibit the histochemical reaction. The controls not only failed, but gave a marked positive reaction in certain areas of the tissue, heavier than in the accompanying test sections. These failures occurred at saccharate concentrations completely effective at higher pH levels. 'Positive controls' of this nature have been obtained with newt liver, mouse liver, and mouse kidney, particularly the latter. For mouse spleen and snail digestive gland, saccharate appears to be an effective inhibitor of the reaction at levels below and above pH 4·0.

It is possible to explain these findings. The normal concentration of ferric ions in the substrate may inhibit the activity of the enzyme to some extent,

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Fig. 1 (plate). The photomicrographs represent sections of the liver of the newt.

A, control section. Note absence of crystalline deposit in both liver-cells and layer of lymphoid-cells. Nuclei stained with Mayer's haemalum.

B, test section. Note the heavy deposit of crystals in the liver-cells, and the absence of them in the lymphoid layer. Nuceli stained with Mayer's haemalum.

C, liver fixed directly in Altmann's fixative and stained by Altmann's technique for mitochondria after paraffin embedding. The mitochondria are filamentous and rod-like in form.

D, liver treated with formaldehyde-saline followed by the normal Altmann fixation, embedding, and staining. The mitochondria are rounded and slightly swollen.

E, liver frozen-sectioned when fresh, fixed in Altmann's fluid, embedded, sectioned, and stained by Altmann's technique. Note the swollen and rounded mitochondria and the gross cracks in the material.

F, liver frozen-sectioned when fresh, incubated in acetate buffer, and then submitted to the Altmann procedure. Note the absence of mitochondria. The appearance of the cells closely corresponds to that seen after fixation with chromic acid (see Casselman, 1955).

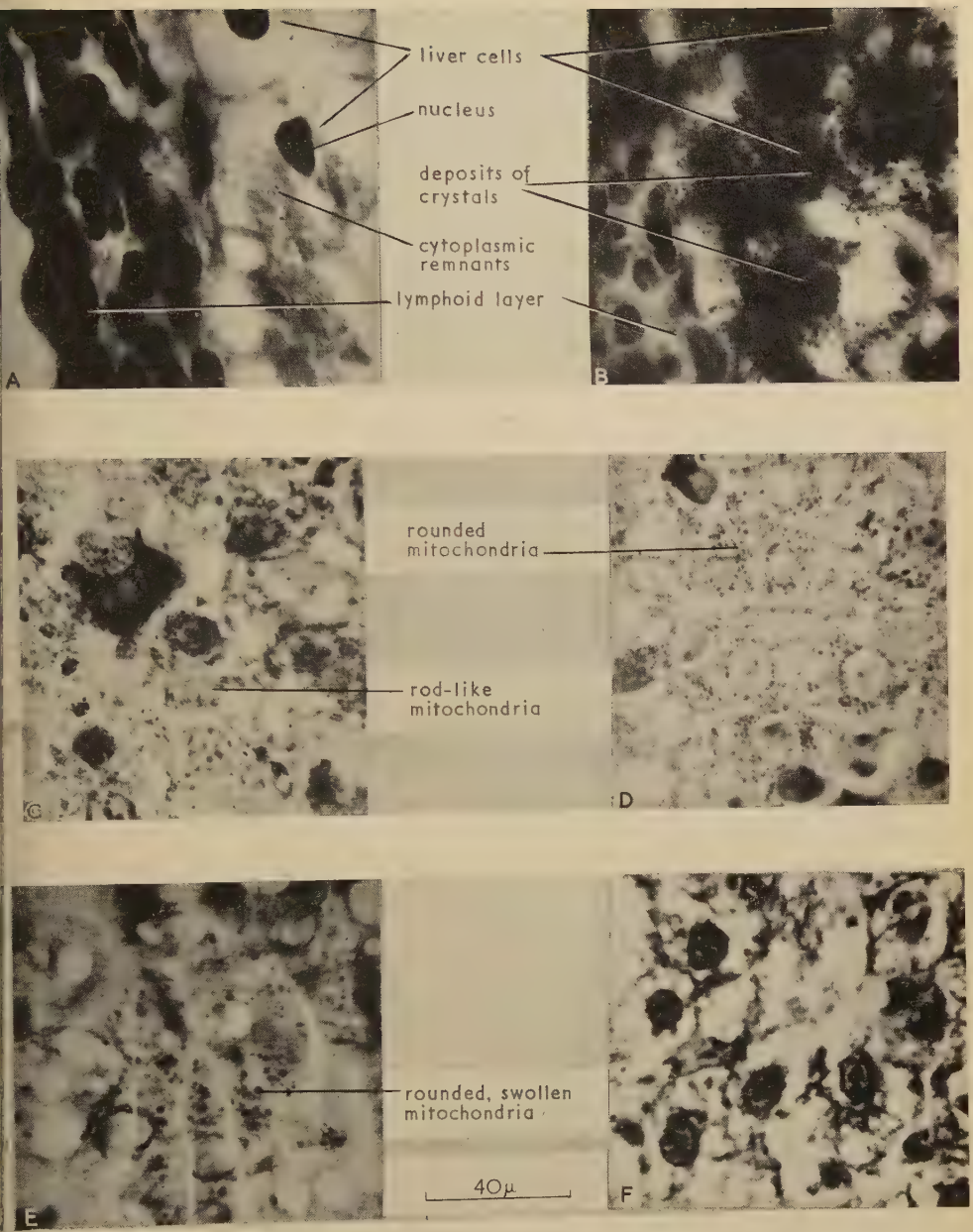

FIG. 1

F. BILLETT *and* S. M. McGEE-RUSSELL

especially at low levels of pH when the concentration is apparently higher. Iron has been said to inhibit glucuronidase (Pearse, 1954). The saccharate added to the controls would appear to react with the ferric ions (see previous paper, p. 39). This reaction could lead to a reduced concentration of both inhibitors, and give control sections in which the enzyme is apparently more active than in the test sections incubated simultaneously.

A further complicating factor is that the tissue itself may act as an adsorption surface upon which ferric 8-hydroxyquinoline is taken up. The adsorption is shown by the darkening which may occur in sections placed in substrate, and the accompanying lightening in colour of the substrate mixture. This is noticeable with sections of *Helix* digestive gland. Different tissues, after fixation, show noticeable differences in their ability to take up ferric iron (Wigglesworth, 1952). It is to be expected, therefore, that a uniform substrate mixture need not *necessarily* give identical reactions upon incubation with different tissues, even if they contain comparable activities of β-glucuronidase. The different amounts of ferric 8-hydroxyquinoline adsorbed from the substrate mixture by different tissues might have a direct effect upon the activity of the enzyme, through the consequent variation in the concentration of ferric ions. In addition, a decrease in the amount of ferric 8-hydroxyquinoline through adsorption may mean that a longer time is required to establish conditions suitable for rapid precipitation of crystals, through the initial enzymic hydrolysis. Optimal conditions are subject, clearly, to the effects of a large number of variables apart from the activity of the enzyme.

The effect of certain stages of the technique upon mitochondria

It has been demonstrated many times that the mitochondria of tissues are easily destroyed by mild reagents, including dilute acetic acid. The following experiments were designed to determine the effect of the formaldehyde fixation and the incubation in acetate buffer upon the mitochondria of newt liver-cells. After the treatments with formalin, and acetate buffer, the tissue was subjected to Altmann's standard procedure for the preservation and staining of mitochondria (Altmann, 1890). Control pieces of tissue fixed immediately in Altmann's fixative, without any prior treatment, were compared with the experimental material.

Formaldehyde fixation. Two small pieces of the liver of *T. cristatus* were cut from the entire organ. The pieces were about 5 mm square, and of the thickness of the liver. One piece was placed directly into Altmann's fixative, the other was fixed in ice-cold formaldehyde-saline for 4 hours, washed in saline, and then placed in Altmann's fixative. After 24 hours in the osmium-dichromate mixture, both pieces of tissue were embedded in paraffin and sectioned at 3 μ, and stained with Altmann's acid fuchsin. The untreated tissue has cells packed with rod-like and rounded bodies, deeply stained. The tissue treated with formaldehyde also shows mitochondria, but these are more rounded and swollen. The marked swelling effect of concentrations of formaldehyde below 5% upon living cells has been noted by Crawford and Barer (1951).

Acetate buffer. Fresh newt liver was placed on the stage of a freezing microtome and frozen. Sections were cut at 200 μ. Some of these sections were placed directly into Altmann's fixative, others were placed in 0·1 N acetate buffer (pH 5·2) and incubated at 37° C for 6 hours, washed in distilled water, and then placed in the fixative. After 24 hours' fixation, these thick sections were embedded in paraffin, and sections at 3 μ were cut at right angles to the original plane of sectioning. The normal Altmann staining technique was then applied to them.

Examination revealed that the mitochondria had suffered some damage in both procedures (fig. 1, E and F). The tissue which had been subjected to freezing-sectioning at 200 μ showed mitochondria mainly in the form of rounded granules instead of the rod-like bodies of the typical preparations. Sections subjected to the acetate buffer in addition to the frozen-sectioning showed only a few rounded bodies, apparently the remains of mitochondria.

The effect of formaldehyde fixation upon the activity of the enzyme. As measured by their ability to catalyse the hydrolysis of quinolyl-8-glucuronide, fresh newt liver extracts appear to be 3–4 times as active as fresh mouse liver extracts, and to possess about one-quarter the activity of extracts of the digestive gland of *Helix pomatia.* Fixation in ice-cold 4% formaldehyde for 3 hours reduces this activity by 30-40% in all three tissues (Billett, unpublished observations). Thus the finding of Seligman and others (1951) that rat liver enzyme is almost insensitive to fixation by cold formaldehyde may not be of general application.

Discussion

The modified technique for the localization of β-glucuronidase described for the snail digestive gland appears to work well when applied to newt liver. Application of the technique to mouse spleen, liver, and kidney has been disappointing. Of the three mouse tissues, kidney gives the best reaction, and a localization of the enzyme similar to that described by previous authors (Friedenwald and Becker, 1948; Burton and Pearse, 1952) is indicated. In the case of mammalian tissues we have not been able to obtain the fine intracellular localization which other authors have described when using the Prussian blue method (Campbell, 1949). In the case of newt liver the crystals of ferric 8-hydroxyquinoline which form are of about the same size as the mitochondria, but they do not appear to be associated with them.

Preparations made by the Altmann technique indicate a certain amount of damage to the mitochondria, after formaldehyde fixation and incubation in acetate buffer. The pre-treatment may alter the tissue in such a way that the mitochondria are no longer stained by the acid fuchsin, but this appears unlikely. No unstained bodies of mitochondrial type are visible with the phase-contrast microscope. Bodies which are probably distorted mitochondria, or fragments of them, are stained by the acid fuchsin. The incubation technique would appear to produce considerable distortions of the cytoplasm.

It is thus reasonable to conclude that fine intracellular localization of

β-glucuronidase is not possible with the present forms of the ferric 8-hydroxyquinoline method. The grounds are, first, that the precipitate indicative of the site of the enzyme is too coarse, and secondly, that the necessary pre-treatment involves an inevitable amount of destruction of the fine structure of the cytoplasm.

We wish to thank Professor A. C. Hardy, F.R.S., for the facilities which he provided for us within his department. We were greatly helped in our work by the encouragement and interest of Dr. J. R. Baker. This work was carried on during the tenure of a personal grant from the Medical Research Council (F. B.) and of a Christopher Welch Scholarship in the University of Oxford with support from the Department of Scientific and Industrial Research (S. M. M.-R.).

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The Histochemical Recognition of Phenols, especially Tyrosine

By JOHN R. BAKER

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SUMMARY

Sections of tissue embedded in collodion are heated in an acidified aqueous solution of mercuric sulphate and sodium nitrite. A red colour denotes the presence of phenols (generally the tyrosine of proteins). The reagent is more rational than Millon's and most of the modifications of his method used up till now in histochemistry. It gives a particularly strong coloration.

THE purpose of the work described here was to find a test for phenols that would be more sensitive, dependable, and rational than Millon's (1849) and the various modifications of his method that have already been introduced into histochemistry. Of these, the test of Bensley and Gersh (1933) is probably the most often used; but it was used by them for frozen-dried tissues, and it generally gives a feeble colour with sections of fixed material.

It was shown by Meyer (1864) that a red colour is produced when tyrosine is treated with mercuric chloride in the presence of potassium nitrite in acid solution. Millon's test depends in reality on a similar reaction, nitrite being formed in the preparation of the reagent. The various authors who have introduced tests resembling Meyer's to replace Millon's have all used sodium or potasium nitrite, but they have worked with different salts of mercury. Lintner (1900) used mercuric nitrate, Nasse (1901) the acetate, and Folin and Ciocalteu (1927) the sulphate. The very sensitive test devised by the latter authors was perfected and used quantitatively by Folin and Marenzi (1929).

Mirsky and Pollister (1946) used mercuric sulphate, sulphuric acid, and sodium nitrite as a test for tyrosine in their study of 'chromosin'. They found that their reagent dissolved histone from their material, which had not undergone previous fixation. To prevent this, Pollister and Ris (1947) used trichloracetic acid instead of sulphuric.

It is claimed that in all these tests the reaction proceeds in two stages (Vaubel, 1900; Gibbs, 1927). A nitrosophenol is formed first, by the substitution of -NO for -H in a position *ortho-* or *meta-* to the hydroxyl of the phenol. The red compound is then formed, apparently by the inclusion of mercury in a new ring that also includes the nitrogen of the nitroso-group. Nearly all phenols, except those that are doubly substituted in the *ortho* or *meta* positions, react positively, though thymol provides a partial exception. Tyrosine is naturally positive.

I applied both Nasse's and Folin's reagents to sections of fixed tissues, and obtained a much stronger reaction with the latter's. I then set out to perfect a histochemical test for phenols, based on Folin's method.

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Experiment showed that particularly strong coloration was given after fixation by formaldehyde, but other common fixatives may be used: Zenker, for instance, or Heidenhain's 'Susa', or Brasil. Susa commends itself because it contains trichloracetic acid, but the colour is not so intense as when formaldehyde is used alone. Helly is unsuitable, because it produces pieces of tissue that react negatively in their outer parts. Even if the fixative used contained mercuric chloride, the section should not be treated with iodine solution.

The concentrations of mercuric sulphate, sulphuric acid, and sodium nitrite that were necessary to give strong coloration with sections of fixed tissue were discovered by empirical experiment.

When a section was heated in the reagent, it generally began to become coloured at about 50° C. Intensification of the colour was especially rapid above 90° C. Excellent results as regards colour were obtained if sections were boiled in the reagent, but this is likely to cause damage with frozen or paraffin sections. It was found, however, that when tissues were embedded in collodion, they could be subjected to quite violent treatment without being injured. The exact nature of the collodion used is of no importance. Low-viscosity nitrocellulose works well. It is strange that collodion sections have so seldom been used in histochemistry.

The colour produced by the reaction is permanent in some mounting media, but fades gradually in Farrants' medium, Canada balsam, and those glycerine-jellies that are neutral or nearly so in reaction. There is no fading with Kaiser's (1880) glycerine jelly (pH 5·5). The phenol in this medium in no way disturbs the result of the test.

These solutions are required:

Formaldehyde-saline. The following solution is suitable:—

Formalin	10 ml
Distilled water	83 ml
Sodium chloride, 10% aq. . .	7 ml

Chalk or marble may be kept in the solution.

Mercuric sulphate solution. Add 10 ml of concentrated sulphuric acid to 90 ml of distilled water. Add 10 g of mercuric sulphate. Heat till dissolved. Cool. Make up to 200 ml with distilled water. The solution is stable.

Sodium nitrite solution, 0·25% aqueous.

(1) Fix the piece of tissue overnight in formaldehyde-saline.

(2) Embed in collodion.

(3) Cut sections at 20–30 μ (or less, for special purposes). Sections may be stored indefinitely in 70 or 80% alcohol.

(4) Bring a section through 50% alcohol to distilled water.

(5) Put 5 ml of the mercuric sulphate solution in a small (50 ml) beaker. Add 0·5 ml of the sodium nitrite solution.

(6) Put the section in the beaker and heat it gently over asbestos gauze until the fluid just boils.

(7) At once remove the beaker from the gauze and allow the fluid to cool somewhat for 2 or 3 minutes.

(8) Wash the section in three lots (about 50 ml each) of distilled water. (Tap-water is unsuitable.) Leave the section at least 2 minutes in each wash. (Long periods are not harmful, as the coloured reaction-product is insoluble.)

The section may now be mounted in any one of several ways. Pure glycerine is a very suitable medium. To mount in Kaiser's glycerine-jelly, pass through 50% glycerine. One can easily bring a section right through the test and mount in glycerine-jelly in 20 minutes. Alternatively one may bring the section up through the alcohols to 95% and then mount in euparal, which dissolves the collodion (see Sheppard, 1921); or one may pass from 95% alcohol through creosote or origanum oil into DPX (Kirkpatrick and Lendrum, 1939).

In the finished preparation phenols are recognized by the red, pink, or yellowish-red colour-reaction. In the tissues of animals the reaction will be due in the great majority of cases to the presence of tyrosine as a constituent of protein.

An excellent object on which to learn the technique is the duodenum of the rabbit, cut out in such a way as to include a part of the pancreas. The part of the duodenum near the opening of the pancreatic duct is the most suitable. The very strong positive reaction in the zymogen granules of the pancreas and in the secretion-granules of certain cells of Brunner's glands is at once noticeable. These cells are the ones called 'dark' by Tschassownikov (1928), on account of the strong staining of the basal region of the cell by iron haematoxylin. The resemblance of these cells to the exocrine cells of the pancreas was remarked by Tschassownikov, and the reaction to this test for phenols provides additional evidence of similarity. These cells, which are especially abundant in the rabbit and hare, appear to deserve further study. The ground cytoplasm and nuclei of the various cells in the section are less strongly coloured than these secretion-granules.

Collagen is almost or quite colourless, because the amount of tyrosine in this protein is so very small. A striking pair of preparations may be made from any organ (such as the mammalian vas deferens) which has a thick layer of collagen fibres lying between components that react positively to the test for phenols. If one section of such an organ be stained by a method for collagen and the other by the test for phenols, the one gives almost a negative image of the other.

I thank Miss B. M. Jordan for practical help.

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A Demonstration of Dehydrogenase Activity in the Mitochondria of Living Cells

By ARTHUR HUGHES

(*From the Anatomy School, Cambridge*)

With one plate (fig. 1)

SUMMARY

A method is described whereby fresh specimens of neural tissues are examined in squashes under the phase microscope. When squashed in contact with the sensitive tetrazolium derivative known as 'INT', granules of formazan are precipitated around the mitochondria within the neurocytoplasm. Application of these techniques to squashes of the spinal cord of the ray has given a direct demonstration of the dehydrogenase activity of mitochondria within fibres of the white matter of the cord.

METHODS for the study and detection of intracellular enzymes have been classified by Pearse (1953, p. 216) into three groups. Of these, the first, that of 'purely biochemical homogenization methods' in which cells are mechanically disintegrated with minimal breakdown of their constituent inclusions, has so far yielded the greater part of our present knowledge concerning the function of mitochondria. Bensley and Hoerr in 1934 described a method for the isolation of apparently intact mitochondria from the liver of the guinea-pig, but it was not until the 1940's that Claude (1941, 1944) began to separate tissue homogenates by differential centrifugation into fractions each containing particles of one order of size, and to examine the enzymes associated with each fraction, and thus with the various sizes of particle. One of the main conclusions which emerged from these researches was that the important respiratory enzyme succinic dehydrogenase is associated with mitochondria. Work of this kind has been further developed mainly by Schneider, who in a recent review (1953) lists over thirty enzyme systems which are now known to be present in these bodies.

Homogenization methods are not without possible objections, and without the precautions which are observed by Schneider and his colleagues are liable to give misleading results. Other methods of studying the activity of mitochondria are thus by no means superfluous.

The second and third of Pearse's groups of methods are histochemical. In the second, used mainly by Dr. Holter and his colleagues at Copenhagen, adjacent portions or sections of a cell are studied either chemically or histologically. By alternate treatment of such serial isolates throughout the length of a cell the distribution of enzymes and of mitochondria can thus be compared. The third group is that of histochemical methods in which a coloration is developed within a cell under the influence of its enzymes. The vital staining of mitochondria with Janus green, which was observed by L. Michaelis as far back as 1900, has in recent years been linked with their oxidative activity by

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Lazarow and Cooperstein (1950, 1953), though other but perhaps less plausible explanations of their affinity for vital dyes have been advanced (Marston, 1923). Other dyes, of sufficiently low toxicity to be applied to living cells, have also been used to study oxidation-reduction systems in mitochondria (Brenner, 1949). In recent years, however, the main development in the histochemical study of the dehydrogenases has been the introduction of the tetrazolium group of compounds. The early history of the use of these substances as biological reagents is described by Smith (1951) and by Pearse (1953). Tetrazolium salts are pale-yellow soluble compounds which are readily reduced to a corresponding insoluble and strongly coloured derivative known as formazan. Lakon (1942) showed that this reduction by plant-cells was an enzymatic process, which in homogenates of rat tissues was shown by Kun and Abood (1949) to be due to succinic dehydrogenase. A number of tetrazolium derivatives of different sensitivity have been prepared, and have been tested on various tissues; of these compounds, Rogers (1953) has concluded that on mammalian skin 2-(*p*-iodophenol)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium, known as iodo-nitro-tetrazolium or INT, is particularly effective. This compound has also been used by Pearson and Defendi (1954) and by Defendi and Pearson (1955) in some recent studies on various tissues of the rat. Rogers has found that the non-enzymic reduction of INT by sulphydryl groups can be readily distinguished from the action of dehydrogenases thereon because only the latter rapidly produce a granular precipitate. A number of substrates which are known to enter the tri-carboxylic acid cycle were added to slices of skin in the presence of INT, and so in experiments where reduction occurred, the presence of the corresponding dehydrogenases was revealed.

Hitherto, positive results with tetrazolium salts have been inferred only in the presence of an aggregation of granules of formazan sufficiently dense to be seen under moderate powers of the microscope. This has meant that any association with the presence of mitochondria has been obscured. In work of this kind, moreover, fresh tissues have usually been sectioned by means of the freezing microtome, a procedure which greatly distorts cytoplasmic structure. Thus tetrazolium compounds have hitherto been used more as histochemical than as cytochemical reagents.

The present paper describes means by which a tetrazolium salt may be used to demonstrate the localization of dehydrogenase activity within an intact cell. It was found that the cells within living cultures of chick embryonic tissues in hanging-drop preparations showed no unfavourable reaction to treatment with INT at concentrations not higher than 0·25%, and that under the phase microscope with an immersion lens, one could follow the precipitation of individual granules of formazan around mitochondria. In a fibroblast the precipitated formazan was confined to the region of the cell where these inclusions were most abundant, but their continuous slow movement soon obscured the distribution of granules of formazan in relation to individual mitochondria. However, in nerve fibres within the outgrowth of cultures of

ragments of the nervous system, the mitochondria are widely separate, and n association exclusively between them and the precipitated formazan could e precisely demonstrated.

In most of the experiments described in this paper, the procedure described y Rogers (1953) has been followed, in that the living cells were treated with a nixture of equal volumes of 0·5% INT and 0·2 m sodium succinate, diluted o five volumes with buffered saline.

Another way in which unfixed tissues can be prepared for microscopical xamination is by squashing or spreading. This method was used by Valentin n 1836 in one of the very earliest studies on the microscopical anatomy of the ervous system after the introduction of the achromatic compound micro-cope (Baker, 1949). It is still a valuable means of studying the living neurone, nd of the effect of reagents thereon (Hughes, 1954). Recently it has been ound that if dorsal root ganglia dissected from chick embryos in the second nd third weeks of development are squashed in the buffered INTsuccinate nixture, then incubation at 37° C for 15 minutes will reveal the general listribution of succinic dehydrogenase activity within the perikarya of the eurones. A study is in progress of the development of this enzyme system luring embryonic life by treatment of spreads of dorsal root ganglia with everal tetrazolium derivatives of varying sensitivity. In these neurones, how-ever, the mitochondria are densely packed and extremely small. The electron nicroscope reveals that their average diameter is about 0·1 μ. Here therefore one is not able to demonstrate any individual relationship between mito-chondria and granules of formazan. In squashes of the nervous system from another source, however, this has been found possible.

Recently, during visits to the laboratory of the Marine Biological Associa-tion, the squash technique has been applied to the spinal cord of an elasmo-branch fish. Living rays of a number of species, mainly the thornback, *Raia clavata*, of various ages are available at Plymouth. They range from young specimens some 3 in. across, early in the first year of growth (Clark, 1922; Steven, 1936), to large individuals several years old, which may measure 2 feet across the disk.

In these fishes it is possible very rapidly to make spreads of the fresh spinal cord. After the cord of a living ray has been severed just behind the brain, a cut parallel to the surface of the disk through the neural arches reveals a considerable length of the cord. It is then divided into fragments of suitable size, each of which is squashed between a slide and coverslip (fig. 1, A–D).

In such preparations under the phase microscope there can be seen both cell-bodies and fibres. In both, the texture of the cytoplasm with its various inclusions can be studied. As previous workers have concluded (de Renyi, 1931), uninjured neurocytoplasm is usually a gel. In squashes of the spinal cord of the ray, the microscope shows that the granular contents of the neurones are free from all detectable Brownian movement, in contrast with, for instance, the behaviour of the vacuoles within the cytoplasm of the ery-throcytes. In the neurones of these preparations, both in cells and fibres,

Brownian motion is an early sign of cytolysis, the absence of which is thus a check on the physiological state of the material. Judged by this criterion, it remains free of any sign of disintegration for at least 30 minutes, a period which allows ample time for a series of photomicrographs within the preparation. These were taken by phase-contrast in the way described in Hughes (1954), with an exposure of 1–2 seconds at a magnification of ×400 on 35 mm film.

In specimens from the cord of a young individual, myelination is still in relatively early stages. Under the phase microscope, cells of the ventral horn can readily be identified in such squashes, and their cytoplasmic texture photographed and studied. A study is in progress of the contents of these cells by the aid of this method and of others. In spreads of the cord of more mature individuals, the field consists so largely of myelinic material of relatively high refractive index that the cell bodies are largely obscured. However, the larger fibres of the white matter, bordered by disordered myelin, are still conspicuous. They are up to 12 μ in diameter, and at fairly regular intervals within them there can be seen narrow streaks of refractile material. These may be identified as mitochondria. They are about 3 μ in length; there may be as many as five rows of them across the width of a fibre. The distance between the mitochondria increases with the age of the individual. Owing to the consistency of the surrounding cytoplasm, they remain constant in position (fig. 1, A).

The reaction of these mitochondria with INT may readily be demonstrated. The reaction of the nervous system of the ray with this reagent is much stronger than it is with neural tissues of the chick embryo. Within a minute of the spreading of fragments of the cord of the ray in the buffered INT/succinate mixture, formazan granules are seen at the periphery of the squashed tissue, which after a few minutes at room temperature are dense enough to give a pink margin, visible by direct inspection. After 20 minutes, formazan granules are present throughout the spread tissue, both in cell-bodies and in fibres. Under the phase microscope, granules within cells can at once be distinguished from those outside, because the latter alone exhibit Brownian motion. In the cell-bodies of neurones, the cytoplasm becomes so densely packed with formazan granules that a general coloration within them is visible under low powers of the microscope. No granules were ever seen to be within a nucleus.

In the large fibres within these preparations, after treatment with the INT mixture, short rows of formazan granules were formed parallel to the axis of

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FIG. 1 (plate). The four figures are phase-contrast photomicrographs from squashes of the spinal cord of *Raja* spp. at different ages, showing some of the larger fibres of the white matter. Disorientated myelin borders the fibres on each side. A is from an untreated preparation, and shows the arrangement of mitochondria within the fibre; B–D are from squashes treated with the INT/succinate buffer mixture, and show the precipitation of granules of formazan along the mitochondria. (A is from *Raja naevus*, 20 in. across. The others are from *R. clavata*; B and C from a specimen 12 in. across, D from one 6 in. across.)

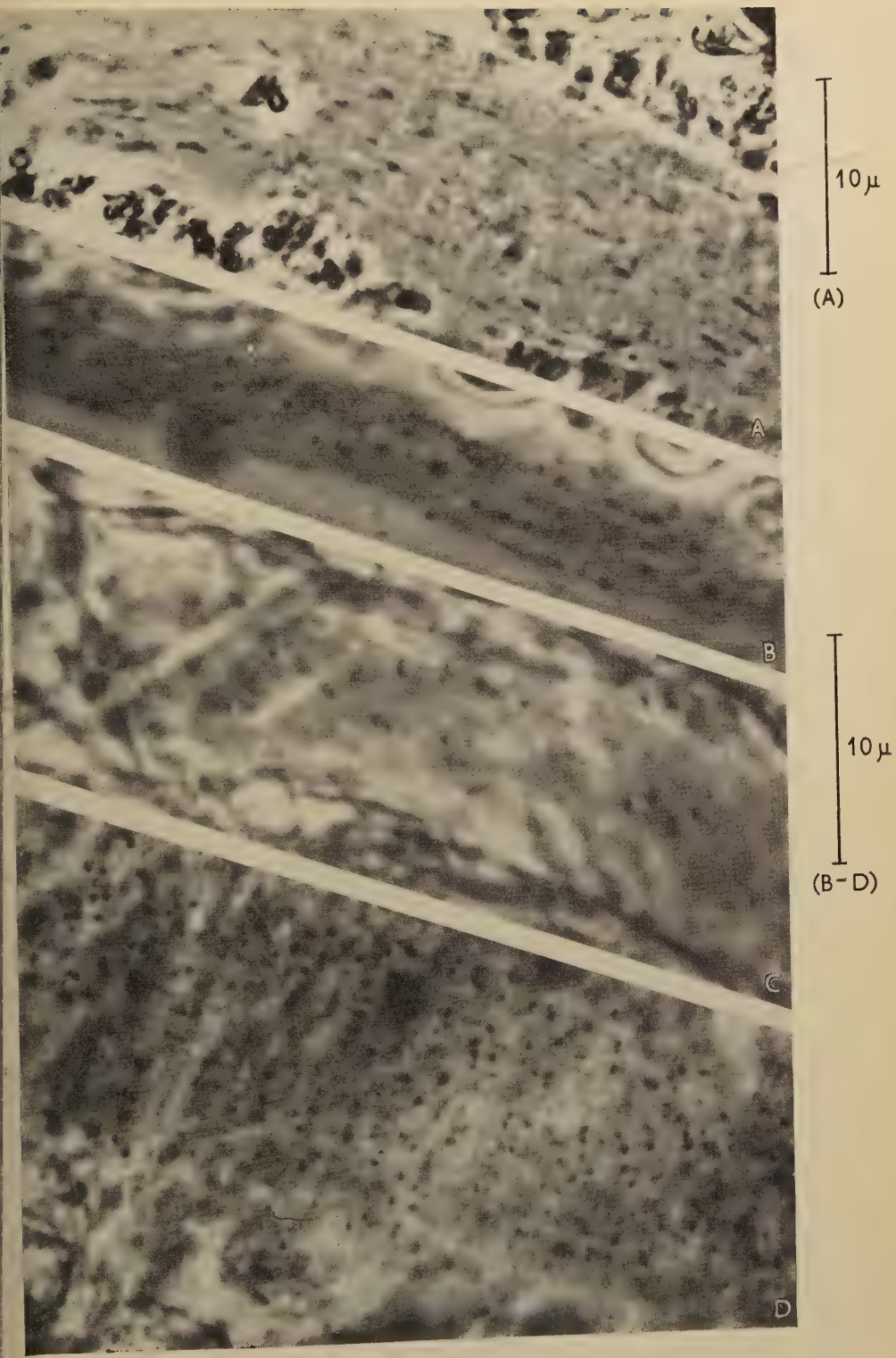

FIG. 1

A. HUGHES

ne fibre, with a general distribution identical with that of the mitochondria ig. 1, B–D). The distance between these rows of granules varied with the age f the individual in the same way as do the mitochondria. Their relationship ith these granules is thus at its clearest in preparations from the largest fish.)ften thin mitochondrial lines could be seen between the granules of a single ow.

The precipitation of granules of formazan in contact with living mitohondria which has here been described may be regarded as a direct cytohemical demonstration of their dehydrogenase activity. This could not be efinitely ascribed to succinic dehydrogenase itself without showing that, in he absence of endogenous substrate, formazan only appeared on the addition f succinic acid. Although under the conditions of these experiments it was ot found possible to wash intact cells free of endogenous substrate without llowing them to begin cytolysis, yet there was no doubt that the reactions ere described with INT were much weaker in the absence of the sodium uccinate of the test-mixture. When a series of fragments of the spinal cord f the ray were placed in equal concentrations of buffered INT, the coloration as always much stronger when sodium succinate was present.

The importance of INT as a test for the activity of mitochondria in an ntact cell was emphasized by comparison with the failure of attempts to tain them with Janus green in squashes of the spinal cord of the ray. Granules eleased from cells which had undergone cytolysis took up the colour at once, ut in a series of experiments it was never observed that sufficient dye diffused hrough the gelated cytoplasm of an intact neurone to give a recognizable tint o the mitochondria. Because of its sensitivity and of the non-diffusibility of he reaction-product, INT may be regarded as a vital stain for mitochondria with special properties of great value.

I am deeply grateful to Mr. G. E. Rogers for introducing me to the use of INT, for allowing me to use his valuable stock of the material, and for instructng me in its use. I further wish to thank the Director and Staff of the Marine Biological Association for their unfailing kindness to me at Plymouth. The expenses of the work were covered by a grant of the Nuffield Foundation to he Anatomy School, Cambridge.

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The Morphology of the Golgi Bodies with reference to ecretion in the Liver-cells of the Slug, *Anadenus altivagus*, as seen under the phase-contrast Microscope

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With one plate (fig. 1)

Summary

Golgi bodies in the living liver-cells of the slug, *Anadenus altivagus*, exist in two orms: (*a*) homogeneous granules or spheres of dark contrast, and (*b*) spheres showing duplex structure with a light greyish internum and a dark externum, which may be ingle or composite. The greyish internum of these duplex spheroids grows into the ecretory granules, the dark externum disappearing in the process of growth. Mitohondria appear as fibres of light greyish contrast with a dark granule at each tip. This dark granule disassociates itself from the mitochondrion and forms the Golgi ranule of dark contrast—the Golgi 'pre-substance'. The Golgi pre-substance, stainble with neutral red, forms the Golgi spheroids.

Introduction

THE Golgi bodies in the secretory cells of animals have been the subject of investigation since their discovery, but almost invariably cytologists have employed techniques which involve metallic reduction of silver or osmium. But, as has been repeatedly pointed out by Nath (1944, 1956) and Baker (1950, 1953, 1954), not much reliance can be placed on such techniques, which involve long osmication or silver deposition in the tissue. These authors have also pointed out the great importance of the study of the living cell, which has been rendered much easier and more reliable by the recent advances in microscopy. A great deal of work on germ-cells and neurones has been done by phase-contrast microscopy in this laboratory and elsewhere, but living gland-cells do not seem to have attracted much attention.

Hirsch (1939), to whom we owe much of our knowledge of gland-cells, has described two phases of the Golgi bodies in the living pancreas: (1) 'pre-substance', composed of solid granules of Golgi substance showing no differentiation into external and internal regions, and (2) the 'Golgi system', which consists of a chromophil externum and a chromophobe internum. The secretion, according to this author, arises in the internum of the Golgi system.

In view of the above facts the study of the gland-cells of various animals has been undertaken by the author, largely by phase-contrast microscopy. The present studies of the living cells of the liver of the slug, *Anadenus*, have more or less confirmed the findings of Hirsch (1939). In this material, however, the Golgi spheroids seem to be bodily transformed into secretory

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granules, as no Golgi granules representing the separated 'externum' or th 'Golgirest' of Hirsch could be identified amongst the secretory granules.

Similar observations have also been made by the author on the liver-cel of a local snail, the account of which will be published at a later stage.

Material and Technique

The studies of the liver-cells of the slug, *Anadenus altivagus* Theobal were carried out at Simla, Panjab, during the months of August and Sep tember 1955. The liver was removed from the living animals and was place in 0·7% sodium chloride solution, to 100 c.c. of which 0·2 c.c. of 10% calcium chloride solution had been added. The same physiological solutio was also used as the liquid medium for the microscopical preparations.

Neutral red chloride (B.D.H.) and Janus green (Horleco, U.S.A.) wer employed supravitally at the standard concentration of 0·01% in the abov physiological solution. The latter, however, did not prove successful. Th bright and the dark phases of the microscope were used alternately for study ing the action of the vital dyes.

The Carl Zeiss 'W' phase-contrast microscope, fitted with a photochanger was used for the study of the living cells, and the Carl Zeiss micro-refle camera-attachment with Contax 35 mm camera were used for photomicro graphy. All the photographs were taken with a K8X ocular and '1·25/10 oil-immersion objective. The photographs were further enlarged three time and are untouched.

Observations

The youngest cells in the liver of *Anadenus* are comparatively small, round or oval cells generally lying in pairs. The cytoplasm at this stage reveals number of mitochondrial fibrillae, which give light greyish contrast. To eac end of the fibrilla is attached quite a prominent granule showing a very hig phase-change. Some of the mitochondrial fibrillae, however, seemed to b devoid of such granules. In addition to the mitochondria a few dark, separat discrete granules of various sizes were also observed in the cytoplasm, show ing a phase-change equivalent to the tip-granules of the mitochondria an staining with neutral red (fig. 1, A and B). These can be homologized to th 'Golgi pre-substance' of Hirsch (1939).

In a larger cell the nucleus becomes excentric, a position which it maintain from this stage onwards. The mitochondria, which are fibrillar and granular are pushed into the lower region of the cell. In addition to the very smal granules described above, the cell now shows a few Golgi bodies which are i

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Fig. 1 (plate). Photomicrographs of living liver-cells under positive phase-contrast.
A and B, early cells showing mitochondria and Golgi granules.
C, cell showing formation of the composite spheroid.
D and E, cells showing various phases of the Golgi bodies.
F, G, and I, cells showing Golgi bodies and secretory granules.
H, cell showing polarity in the distribution of the Golgi bodies and secretory granules.

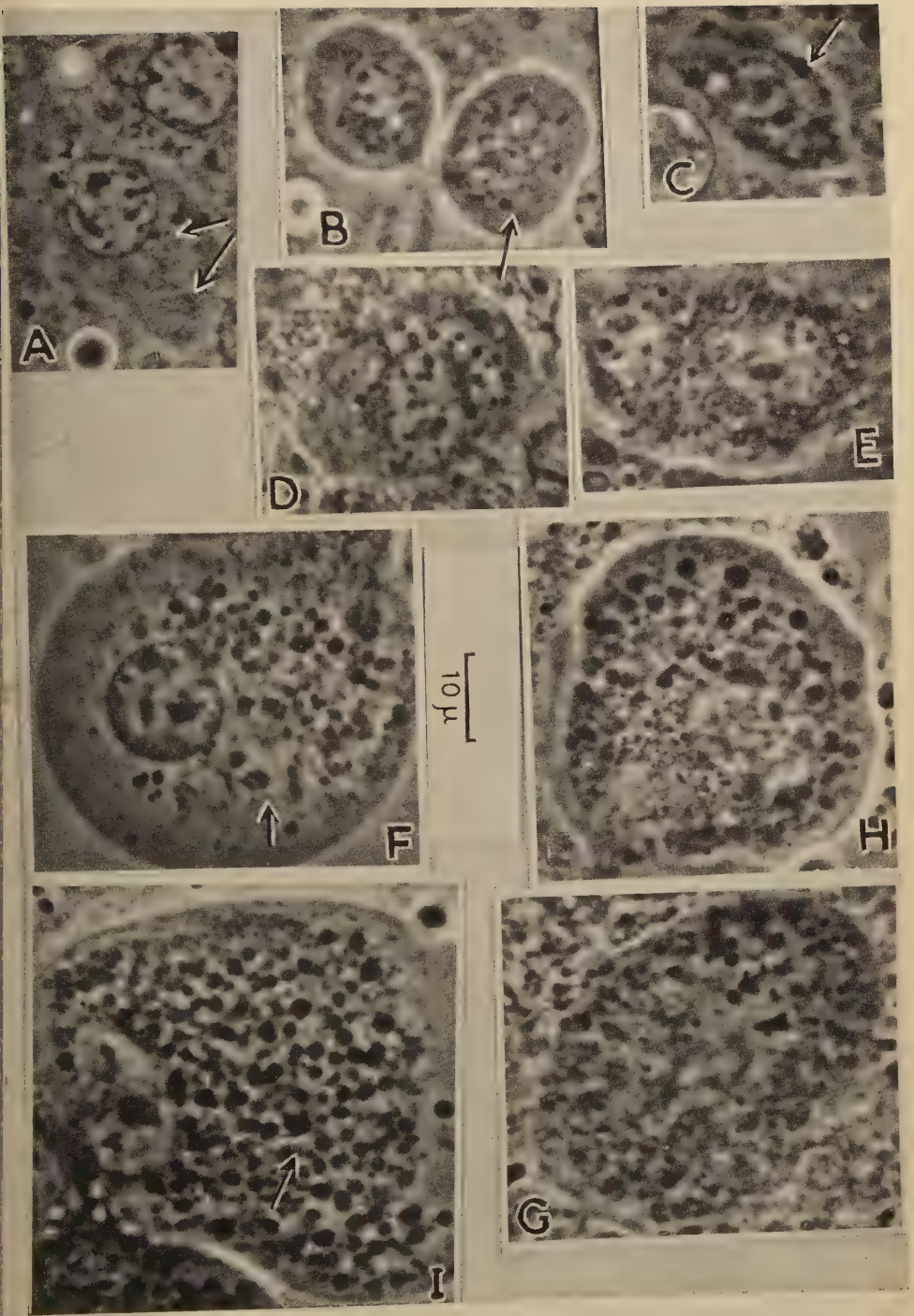

FIG. 2

R. RISHI — PLATE I

e form of large granules, rings, or crescents, the latter showing a duplex ucture (fig. 1, D and E). A critical study of the Golgi rings or crescents, wever, reveals that they are more or less spherical bodies in the form of a eyish sphere of a greyish contrast enclosed completely or partially by a eath of much darker contrast. It appears, therefore, that the rings and escents are optical sections of these Golgi spheres. By changing the focus can be seen that the dark rim of the Golgi ring travels along the surface of e grey sphere; and the Golgi sphere appears as a dark granule both in the permost and lowermost planes. The Golgi elements show a gradual seria- n between the tiny granules of dark contrast and large Golgi spheroids of

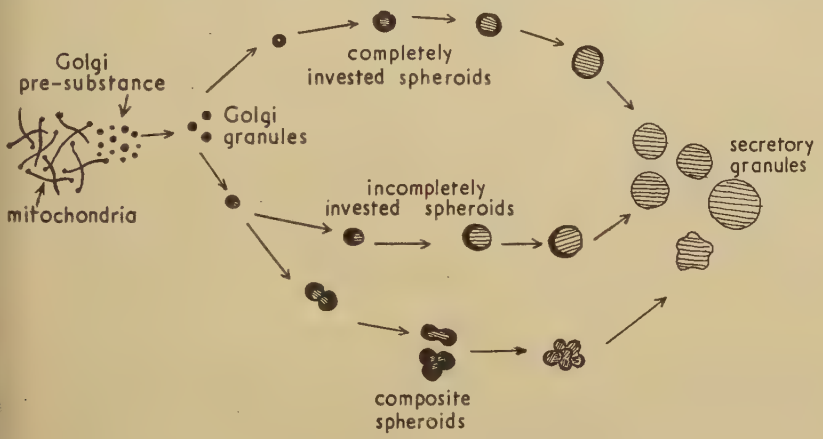

FIG. 2. Diagrammatic representation of the formation of secretory granules and composite spheroids.

uplex structure (fig. 1, D and E). It appears that the tiny granules grow irectly into the latter form.

During the process of growth the tiny dark granules first assume the shape a much darker sphere of a very dark contrast. This sphere, in the majority cases, seems to develop a greyish internum, which grows and forms the ecretion in the older cells. The rim or externum of dark contrast thins out nder the pressure of this growth till ultimately it seems to be absorbed com- letely in the process, leaving behind a secretory granule of large size and reyish contrast. Occasionally, however, the Golgi sphere of dark contrast eems to become crescentic in appearance and develops a greyish sphere in its oncavity. With the growth of this greyish sphere, the crescentic dark sub- ance seems to envelop it partially, thus giving the appearance of a crescent optical sections (fig. 2).

With further growth in the size of the cell the number of the Golgi bodies nd the secretory granules also increases till ultimately the cytoplasm of the ell becomes choked with these bodies, thus completely obliterating the mito- hondria from view (fig. 1, F and G). In addition to the above simple form, the olgi bodies also show a composite structure in which a large irregular sphere

of greyish contrast seems to be surrounded with a number of much dark crescentic caps on all sides (fig. 1, F and I). These composite spheroids ar by the union of individual crescentic forms of the earlier cells (fig. 2). Su a fusion has actually been observed by the author under phase-contr (fig. 1, C). These composite spheroids, however, also form the secretion in t normal manner described above, and the dark crescentic caps seem ultimat to be consumed.

With further growth the contents of the cell show a marked polarity their distribution (fig. 1, H). The Golgi bodies are generally crowded on o side of the excentric nucleus, and the secretory granules fill up the oppos end. The smallest Golgi granules are generally found at the extreme nucle end of the cell, whereas the larger ones are near the secretory granules a intermingled with them. It may be pointed out here that the amount of t secretory granules in a cell appears to be inversely proportional to the amou of the Golgi bodies; this supports the direct origin of the former from t latter. In larger cells the mitochondria could not be seen.

The secretory granules, the grey internum of the Golgi spheroids, and t tiny dark granules (Golgi pre-substance) show a great affinity for neutral re but the dark rim of the Golgi spheroids does not seem to take up the dye at a nor do the larger, homogeneously dark spheres.

Discussion

As was pointed out above, not much work has been done on the mo phology of the Golgi bodies in the living gland-cells of animals; but t available data clearly show that cytologists hold two widely different vie on the morphology of these cell inclusions.

Lacy (1954), in his extensive studies of exocrine and endocrine cells of t mammalian pancreas, believes that the Golgi apparatus in these cells 'is system or network of canals or vacuoles, distinct from both lipoidal bodi and mitochondria'. According to this author, 'this system of canals is hom logous with the Golgi apparatus of the vertebrate neurones'. Lacy also clai to have seen such canals in frozen-dried sections and living material. Sim larly, Moussa (1952), Gatenby (1953), and Adamstone and Taylor (195 describe the Golgi material as a canalicular system in living neurones.

Morgan (1953, *a* and *b*), on the other hand, believes in the spheroid nature of the Golgi bodies in the living pancreas of the mouse.

Hirsch (1939), who has made an extensive study of the gland-cells animals, describes two phases of the Golgi bodies in these cells: (1) '*Pra substanz*', which is in the form of separate dark spheres or granules and tak up neutral red and Janus green B, and (2) the '*Golgisystem*', which is compris of a chromophobe internum surrounded by a chromophil externum. T secretion, according to Hirsch, is differentiated in the internum of the Go system.

The present studies of the living liver-cells of *Anadenus altivagus* und the phase-contrast microscope have convinced the author that the Go

bstance in these cells exists in the form of separate granules or spheres. ne small granules, which have a limited range in size, exhibit a very high ase-change under phase-contrast and correspond to the *Praesubstanz* of irsch. But as the Golgi granules become active in the process of the forma- n of the secretion, they begin to show an area of a grey contrast in their terior which, in some cases, is stuck in the concavity on one side of the anule. With further growth in the size of this greyish sphere the dark aterial (corresponding to the *Externum* of Hirsch) spreads over the greyish here (corresponding to the *Internum* of Hirsch) and envelops the latter mpletely or incompletely, thus giving the picture of rings and crescents th a duplex structure in optical section. It seems from the observations the author that the greyish spheres of the Golgi elements grow into the cretory granules. In the process of this transformation, the sheath of dark ntrast seems to be completely lost. This conclusion is further strengthened the fact that both the secretory granules and internum of the Golgi spheres e stainable with neutral red and present the same phase-change under ase-contrast. The amount of the secretory granules is found to be inversely oportional to the amount of the Golgi bodies in the growing cells of the er of *Anadenus*, and this suggests their direct origin from the latter cell- clusions.

It has also been found, and quite commonly too, that a number of incom- etely invested Golgi spheroids come together and the exposed portions of eir grey interna fuse with each other, thus giving rise to the composite heroids. These composite spheroids are homologous with the 'mulberry heroids' described by Thomas (1948) and Cain (1948) in living neurones d with the *Polysystem* of Hirsch (1939). These composite spheroids, how- er, form the secretion in the normal way.

In spite of the most diligent search, the author has not been able to find any ucture in the living liver-cells of *Anadenus* to which the name 'canaliculi' uld be attributed.

Regarding the origin of the Golgi bodies, it may be pointed out that the nstituents of the youngest cells are the mitochondrial filaments possessing dark granule at each tip, and a few dark, separate granules—the Golgi pre- bstance. But with the growth of the cells the number of Golgi granules creases. In the opinion of the author the tip-granules of the mitochondrial aments break off and form the Golgi pre-substance. This view gets support om the fact that some mitochondrial filaments do not possess these dark anules, and that both the tip-granules of the mitochondrial filaments and e Golgi pre-substance present the same phase-change under phase-contrast. he Golgi pre-substance is also stainable with neutral red.

These observations are in accord with the views of Hirsch (1939), who und that in the living pancreas the small granules are formed on the surface the mitochondria. These granules, according to Hirsch, remain in contact th the mitochondria for some time but later become detached, move to- ards the Golgi field, and constitute the Golgi pre-substance.

The origin of the Golgi bodies from mitochondria has also recently be described in both the fixed (Nath and Chopra, 1955) and living (Nath a Gupta, unpublished) male germ-cells of *Anadenus*.

In conclusion I wish to thank Professor Vishwa Nath for suggesting t problem, for providing facilities for the work, and for his ever-ready help a encouragement throughout the study. I also thank Mr. B. L. Gupta for help in photomicrography and a great deal of skilful practical assistance

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The Cytoplasmic Inclusions of the Neurones of Certain Insects

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With one plate (fig. 3)

Summary

A comprehensive study of the thoracic neurones of fifth instar and immature adults f the locust, *Schistocerca gregaria* Forsk., and of adults of the water-bug, *Laccotrephes ubra* Fabr., has been made by employing the latest cytological techniques and phase-ontrast microscopy. The mitochondria are seen as granules stainable in life with Janus reen. Alignment of granules into filamentous mitochondria has also been observed in xed preparations. The Golgi bodies (lipochondria of Shafiq) are sudanophil, osmio-hil, and argentophil spheroids. The bigger spheroids show a duplex structure. There a chromophil, cortical, lipid component, which may be in the form of a complete ing (*Schistocerca*) or in the form of one or two granules or a crescent (*Laccotrephes*), nd a chromophobe medulla stainable with the basic dyes, neutral red and methylene lue. The smaller Golgi bodies in *Schistocerca* show a homogeneous structure. The olgi bodies have not been observed to be engaged in any secretory activity. Neuro-brillae have been observed in the neurones of the insects studied.

Introduction

SHAFIQ (1953, 1954) described sudanophil and osmiophil bodies in the thoracic neurones of the locust, *Locusta migratoria*, as lipochondria.

Earlier Beams and King (1932) had described similar osmiophil and argen-ophil bodies as Golgi material in the nerve-cells of the grasshopper. They ecorded their observations by using Golgi techniques, and came to the con-lusion that the Golgi elements show a double structure, with a thick osmio-hil cortex and an osmiophobe medulla. They believed the cortical component f the double Golgi bodies to be the true homologue of the classical Golgi lement. Beams and others (1953) revised the previous work of Beams and King (1932) by electron microscopy, and doubted the presence of an osmio-hobe medulla in the Golgi body; they could not demonstrate it owing to the pacity of the osmiophil bodies to the electron microscope. But Shafiq (1953) nterprets the osmiophobe substance as the lipochondrion, and the osmiophil odies of Beams and King (1932) as partly, if not entirely, impregnation rtifacts.

Further, Shafiq (1953, 1954) observed that the lipochondria are stainable with the basic vital dyes, neutral red and methylene blue, in contrast with the indings of Beams and King (1932), who regarded the neutral red granules as ggregations of dye particles. Gatenby and others (1953) described the neutral ed granules in the neurones of vertebrates as senility pigment.

It thus becomes clear that there is a good deal of disagreement regarding the

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true form of the Golgi material of nerve-cells. In view of this, I undertook a comprehensive study of the subject at the suggestion of Professor Vishwa Nath.

Material and Technique

The neurones of the thoracic ganglia of the fifth instar and immature adult of the locust, *Schistocerca gregaria* Forsk., and the adults of the water-bug *Laccotrephes rubra* Fabr., were studied. The living animals were dissected in Ringer or in the saline solution recommended by Baker (1944), and the ganglia were taken out immediately for study. The following techniques were employed:

(1) *Phase-contrast microscopy*. The living neurones of both the species were studied in Ringer or saline solution under the phase-contrast microscope, and photomicrographed.

(2) *Vital staining*. The vital dyes, neutral red, methylene blue, and Janus green, were used in very dilute solution in saline solution.

(3) *Golgi techniques*

(*a*) *Kolatchev*. The material fixed in Champy's fluid for 24 hours was osmicated at 37° C for periods varying from 4 to 12 days.

(*b*) *Formaldehyde-osmium*. The ganglia fixed in formaldehyde-saline were osmicated at 37° C in 1% osmium tetroxide for periods varying up to 48 hours as recommended by Baker (1944).

(*c*) *Aoyama*. The ganglia were fixed in Aoyama's fixative, and the material was treated for varying periods in silver nitrate to study the effect of prolonged treatment with silver.

(4) The material was fixed in chrome-osmium, Helly (with post-chroming) or Regaud (with post-chroming). Paraffin sections were stained in iron haematoxylin. Sections of Helly material were also stained in Masson's tricolor stain.

(5) *Sudan colouring*

(*a*) Paraffin sections of material fixed in Helly and post-chromed in a saturated solution of potassium dichromate at 37° C were coloured in a saturated solution of Sudan black in 70% alcohol, and mounted in Farrants' medium (Thomas, 1948).

(*b*) Smears fixed in Flemming-without-acetic were also coloured with the solution of Sudan black.

(*c*) The material fixed in Aoyama's fluid, after treatment with silver nitrate and the reducing mixture, was postchromed in 5% solution of potassium dichromate (Lacy, 1954). The paraffin sections were coloured with Sudan black.

(*d*) Paraffin sections of post-chromed Helly material were also coloured with Sudan IV for the study of triglycerides.

(*e*) Sudan IV was also used on material fixed in formaldehyde-saline. Sections were mounted in glycerine.

(6) Material was fixed in Bouin and Carnoy for control.

Observations

In the neurones of the thoracic ganglia of the two species studied, there did not appear to be any cytological differences between one individual and another at the same phase of the life-cycle, nor between individuals at the different phases that were used in this investigation.

Mitochondria

In both species the mitochondria can best be studied in material that has been fixed in Helly (with post-chroming) or in chrome-osmium, and stained in iron haematoxylin (fig. 1, F and G; fig. 2, D). They are stainable in life with Janus green, though great difficulty is experienced in working with this dye. The mitochondria are seen to be uniformly dispersed throughout the cytoplasm, and there appears to be no definite pattern of dispersal. The mitochondria appear to be very fine granules, but here and there mitochondrial filaments and chains of mitochondrial granules can be seen. Under the phase-contrast microscope, the mitochondria invariably appear in the form of granules forming a greyish background for the more refringent Golgi bodies (fig. 3, A and B).

Golgi Bodies

The term Golgi bodies has been used to denote the classical Golgi substance, which is lipoidal, and the chromophobe substance, if present, associated with the lipoidal substance. Shafiq (1953, 1954) has used the term lipochondria for these bodies in the thoracic neurones of the locust, *Locusta migratoria*.

The Golgi bodies are dispersed in the cytoplasm of the nerve-cells. They are present invariably in all the thoracic neurones of *Schistocerca* and *Laccotrephes*. The size of the cells varies considerably in both species, in accordance with the amount of cytoplasm present. In the smallest neurones the Golgi bodies are mostly confined to the middle of the cell, but in the larger cells they are uniformly spread throughout the cytoplasm. The Golgi bodies are of spheroidal form, varying considerably in size even in the same cell. There appears to be no direct relation between the size of the Golgi spheroids and the size of the cell, for the bigger spheroids are often seen in the smaller cells and the smaller spheroids in the larger cells. The number of the Golgi spheroids is, however, generally less in the smaller cells.

Phase-contrast study

Schistocerca. Great difficulty is experienced in studying the living neurones under the phase-contrast microscope. The contents of the neurones are very dense, and the Golgi bodies seem to have almost the same refractive index as the ground cytoplasm. But when the tissue has been well teased and the cells have been well flattened by pressure on the cover glass, the Golgi bodies can be seen scattered throughout the cytoplasm of the neurones. They vary considerably in size. Under positive phase-contrast the bigger Golgi spheroids show a dark cortex and an almost colourless medulla (fig. 3, A). The cortex of

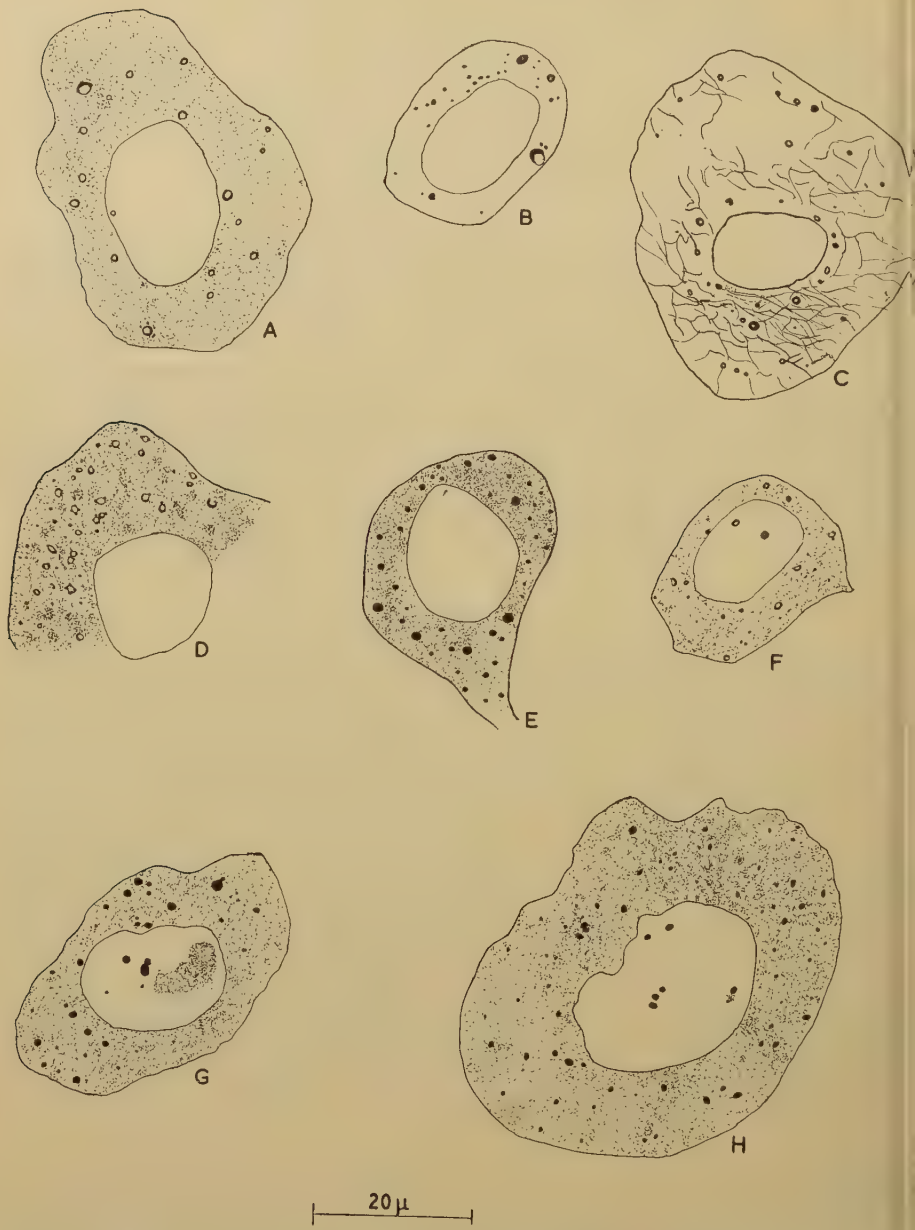

FIG. 1. Neurones of *Schistocerca*. A, fifth instar; Helly/Sudan black. B, fifth instar; from Flemming-without-acetic, Sudan black smear. C, immature adult; Regaud/haematoxylin, showing Golgi bodies and neurofibrillae. D, immature adult; Aoyama (toned), showing Golgi rings and silver deposition. E, immature adult; neutral red. F, fifth instar; Helly/haematoxylin, showing Golgi bodies, and granular mitochondria in the background. G, immature adult; Flemming-without-acetic, haematoxylin, showing Golgi bodies, and granular mitochondria in the background. H, immature adult; Helly/Masson.

the Golgi bodies sometimes appears as a crescent, which must be interpreted as an optical section, for the cortex becomes complete round the medulla when the focus is changed. The smaller Golgi bodies are seen as dark, homogeneous spheroids (fig. 3, A).

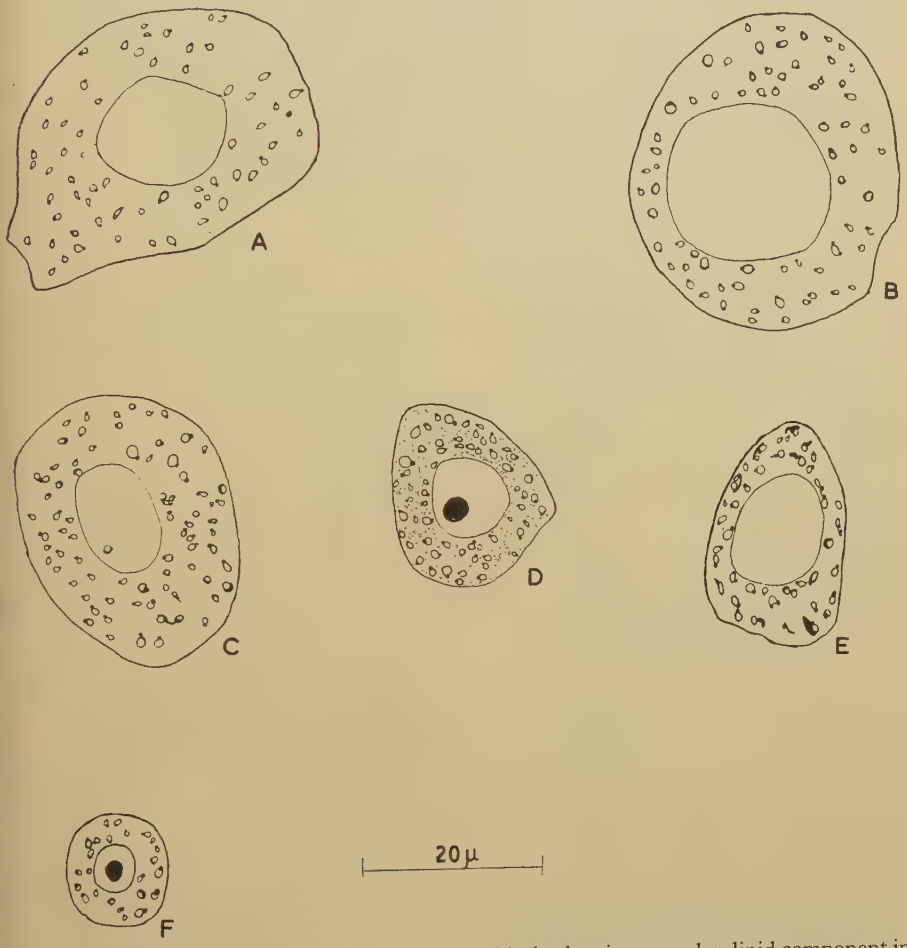

FIG. 2. Neurones of *Laccotrephes*. A, Helly/Sudan black, showing granular, lipid component in association with chromophobe substance. B, smear: Flemming-without-acetic, Sudan black, showing crescents and granules in association with chromophobe substance. C, Aoyama/Sudan black; silver deposition is also seen in addition to the inclusions seen in A and B. D, Champy/haematoxylin, showing granular mitochondria and Golgi bodies. E, Kolatchev 12 days, showing formation of artifacts. F, formaldehyde-osmium, showing optimal impregnation.

With Sudan black techniques the Golgi spheroids show a duplex structure, with a thick sudanophil cortex investing a chromophobe sphere. There is therefore no evidence that the cortex of the Golgi bodies, observed under the phase-contrast, is an optical 'edge-effect', as suggested by Shafiq (1953 and 1954).

Laccotrephes. The cytoplasm of the neurones of *Laccotrephes* is thinner than in *Schistocerca*, and consequently it is easier to study the cell inclusions in the living neurones of this species under the phase-contrast microscope.

In the living neurones of *Laccotrephes* the Golgi bodies are scattered throughout the cytoplasm and there is generally one granule (sometimes two) or a crescent associated with the spheroidal medulla. The cortical granular or crescent-shaped component appears dark, while the medulla is bright under positive phase-contrast (fig. 3, B). Sometimes the rounded shape of the medulla is slightly distorted owing to pressure on the cover-glass.

Vital Staining. Neutral red stains the Golgi bodies brilliantly (figs. 1, E, and 3, G), even when it is used supravitally in very low concentrations. If the ganglia are studied after the dye has acted for about 10 minutes, it is seen that the medulla of the Golgi bodies alone is stained with neutral red. But after about 45 minutes the cortex is not seen and the Golgi bodies become homogeneously stained. This process has been clearly observed with methylene blue in the neurones of *Schistocerca*, and with neutral red in neurones of *Laccotrephes* examined under the phase-contrast microscope (fig. 3, G). Reference should be made to the use of neutral red in similar studies by Thomas (1947), Baker (1949), Shafiq (1953, 1954), and Roque (1954). When the dye is supravitally used it gives a slight tinge to the mitochondria, and sometimes to the nucleus also. Methylene blue stains the chromophobe substance more lightly in *Laccotrephes* than in *Schistocerca* (fig. 3, H). The contents of the chromophobe substance of the Golgi bodies appear to be acidic and watery ('vacuome' of Parat). It is also concluded, in opposition to the findings of Beams and King (1932), that the neutral red bodies are not artifacts.

The Golgi bodies can be demonstrated by short osmication. When living neurones are studied under the ordinary microscope in saline solution to which some osmium tetroxide has been added, the Golgi bodies appear, each with a brownish cortex and colourless medulla.

Chrome-osmium. Helly and Regaud techniques

In chrome-osmium preparations stained with haematoxylin the Golgi bodies are seen as homogeneous spheroids of varying sizes (fig. 1, G) in the neurones of *Schistocerca*. The cortex of the bigger (duplex) Golgi bodies becomes so intensely stained that it conceals the medulla, but in *Laccotrephes* the duplex structure is very clearly seen (fig. 2, D), for the cortical component does not completely ensheath the medulla. Paraffin sections of Helly or

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FIG. 3 (plate). Photomicrographs of neurones.
A, immature adult of *Schistocerca*; living cell, phase-contrast.
B, *Laccotrephes*, living cell, phase-contrast.
C, fifth instar of *Schistocerca*; Kolatchev. Note flattened platelets.
D, the same cell as in fig. 1, D. The arrow indicates a Golgi ring.
E, the same cell as in fig. 1, A. The arrows indicate duplex Golgi bodies.
F, the same cell as in fig. 2, C. The arrow indicates a Golgi body.
G, *Laccotrephes*, living cell, neutral red, phase-contrast.
H, *Laccotrephes*, living cell, methylene blue.

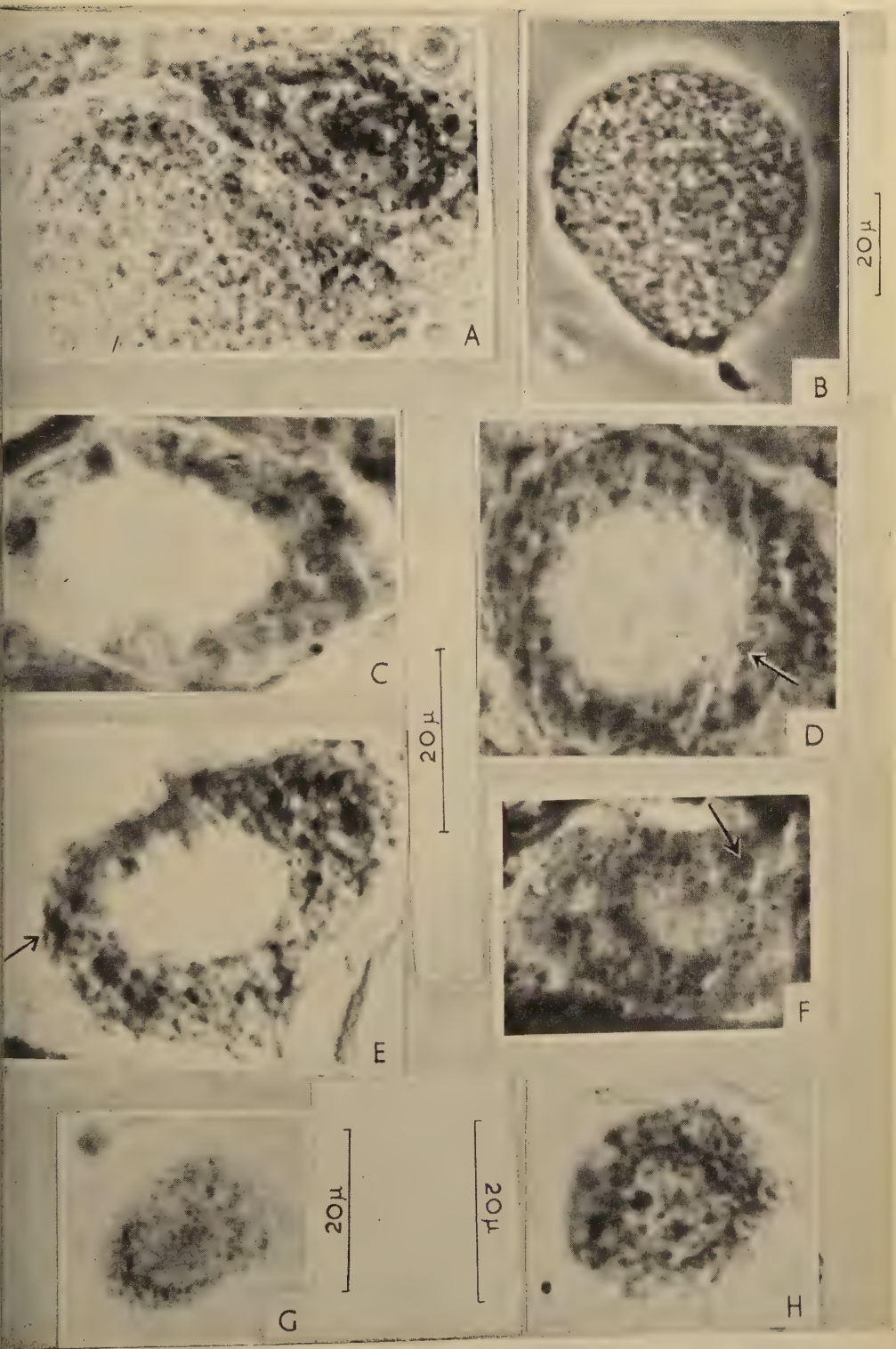

FIG. 3

S. K. MALHOTRA

Regaud material, post-chromed, show duplex Golgi bodies (fig. 1, C and F) in both species after staining with iron haematoxylin. Smaller homogeneous Golgi bodies are always also seen in such preparations. Helly preparations have given especially good results with the neurones of *Schistocerca*, for they show almost the same form of the Golgi bodies as in Sudan black preparations.

The Golgi bodies are also stainable with acid fuchsin, when paraffin sections of Helly material, post-chromed, are stained with Masson's tricolor stain (fig. 1, H).

Golgi techniques

The essential form of the Golgi bodies is preserved in Golgi preparations (Kolatchev and Aoyama), but there is no doubt that these techniques introduce artifacts. In Kolatchev preparations of the neurones of *Schistocerca*, the Golgi bodies show a thick osmiophil cortex and an osmiophobe medulla (fig. 3. C), but the latter becomes greatly swollen up and the Golgi spheroids are converted into flattened platelets. The osmiophil cortex appears thicker in these preparations than with Sudan black, owing to excessive deposition of osmium. So it appears that the osmiophil cortex seen in these preparations is partly an artifact. Shafiq (1953) regards it partly, if not entirely, an osmium deposit. In some of the Kolatchev preparations Golgi crescents and rods are also seen as optical sections of flattened platelets; and some of the rods seem to have been produced by joining up of the smaller spheroids. In Aoyama preparations of the neurones of *Schistocerca* the Golgi bodies show beautifully as rings in the finished slides (figs. 1, D, and 3, D), but invariably artifacts in the form of black granules appear in or round the Golgi rings.

In the neurones of *Laccotrephes* prepared by the Kolatchev and formaldehyde-osmium techniques, the cortical component of the Golgi body appears granular or crescent-shaped and is associated with a clear medulla. There is generally a single granule or a crescent, but sometimes there may be two granules associated with the medulla. This form of the Golgi bodies is only seen when there is optimum impregnation (40 hours) with osmium (fig. 2, F); but if the impregnation is prolonged the cortical component appears bigger. If the impregnation is further prolonged the osmium is deposited all round the medulla and later in the medulla itself (fig. 2, E). Similar artifacts appear in the silver nitrate technique.

Sudan black

The Sudan black preparations show essentially the same form of Golgi bodies as in the techniques described above, or as in the living neurones examined under the phase-contrast microscope.

In the neurones of *Schistocerca* and *Laccotrephes* prepared by the Helly/Sudan black technique, the Golgi bodies appear as heterogeneous spheroids, each showing a sudanophil cortex and a chromophobe medulla (figs. 1, A; 2, A; and 3, E). It has been observed that the cortical component appears smaller than by the long osmication technique. From this it would appear that osmium is deposited on the cortical region of the Golgi body.

Smears of neurones fixed in Flemming-without-acetic and coloured with Sudan black show homogeneously-stained Golgi spheroids in *Schistocerca* (fig. 1, B), but in smears of *Laccotrephes* the double structure can be made out clearly (fig. 2, B). These preparations rarely show an incomplete investing rim to the medulla of the Golgi body in the neurones of *Schistocerca* (fig. 1, B).

Aoyama/Sudan black preparations of neurones of *Laccotrephes* show the cortical component slightly bigger than in other Sudan black preparations, owing to deposition of silver (figs. 2 and 3, F). These preparations show that argentophil bodies are also sudanophil.

Sudan black preparations of *Schistocerca* neurones show, in addition to the bigger duplex Golgi bodies, smaller homogeneously stained Golgi spheroids (figs. 1, A, and 3, E), as seen in Helly's or chrome-osmium/haematoxylin and Helly/tricolor preparations.

The cortical component of the Golgi body appears to be lipid, as it colours with Sudan black. It reduces osmium and silver. It is stained darkly by haematoxylin after chrome-osmium, Helly (post-chromed) or Regaud (post-chromed). It is stainable with acid fuchsin; and it is not seen after Bouin or Carnoy.

Triglycerides seem to be absent from the neurones of insects (*Schistocerca* and *Laccotrephes*), as there is nothing in the neurones that is coloured by Sudan IV.

Neurosecretory products have not been observed in these insects, nor have these Golgi bodies been seen to be engaged in any secretory activity.

Neurofibrillae

Beams and King (1932) and Beams and others (1953) described neurofibrillae in the nerve-cells of the grasshopper, but Shafiq (1953, 1954) did not observe any neurofibrillae in *Locusta*.

My Regaud preparations show very thin filaments interwoven into a net-like structure (fig. 1, C). These appear to be neurofibrillae. The mitochondria cannot be mistaken for neurofibrillae, as the former are completely washed out in Bouin and Carnoy, whereas the latter stand out prominently. Moreover, filamentous mitochondria have not been observed in *Schistocerca* and *Laccotrephes*. The neurofibrillae are more clearly seen in *Schistocerca*. However, they could not be seen in the living material of either species examined under the phase-contrast.

Discussion and Conclusions

The Golgi bodies of the neurones of the locust, *Schistocerca gregaria*, and the water-bug, *Laccotrephes rubra*, exist as spheroids, each showing a duplex structure, a chromophil, sudanophil, osmiophil, argentophil lipid cortex, and a chromophobe medulla. The cortical component may completely ensheath the medulla (*Schistocerca*), or it may be restricted to one or two granules or a crescent, associated with the medulla (*Laccotrephes*). The latter form of the Golgi body is identical with the 'binary spheroids' of *Helix* (Thomas, 1947). The medulla of the Golgi body is not impregnated with osmium or silver

under optimal conditions of impregnation; it is not coloured by Sudan black, but is stainable with the basic vital dyes neutral red and methylene blue. It is thus concluded, in conformity with Shafiq (1953, 1954) and Roque (1954), that the sudanophil and osmiophil bodies exist in association with bodies that stain with neutral red.

The Golgi bodies described in the neurones of *Schistocerca* by the present writer are certainly identical with the lipochondria of Shafiq (1953, 1954); but there are slight differences which are, I think, only differences of interpretation. Shafiq thinks it possible that the lipochondria may be homogeneous bodies. He suggests that the duplex structure of the Golgi body (lipochondrion) as seen under the phase-contrast microscope may be due to an optical 'edge effect', and that the osmiophil cortex may be partly, if not entirely, an osmium deposit. Roque (1954) also describes the duplex structure seen under phase-contrast in the neurones of *Helix* as an optical 'edge effect', although this author describes duplex Golgi bodies (paranuclear bodies), in conformity with Nath (1955), in the sperm-forming cells of *Helix*. Shafiq (1954) says: 'By positive phase-contrast they sometimes appear to be binary in structure, having an outer, dark cortex and a lighter inner medulla; but as with the lipochondria of nerve-cells, it is not possible to assert definitely whether this is an optical illusion or not.'

Beams and King (1932) also described duplex Golgi bodies in the neurones of the grasshopper. But Beams and others (1953) could not clearly demonstrate the osmiophobe substance in the Golgi bodies in the neurones of the grasshopper in their studies with the electron microscope. They doubted the existence of the osmiophobe substance, as 'the Golgi bodies are relatively opaque to the electrons, but they do not seem to be completely homogeneous as is evidenced by the lighter appearing areas within them'.

My observations, however, lead to the conclusion that the Golgi bodies have a duplex structure in the neurones of insects. I am in agreement with Shafiq (1953) and Baker (1954) that osmium first deposits on the lipoidal bodies, then around them, and finally on other structures. I have observed the effect of osmication on the material initially fixed in Champy or formaldehyde-saline. The material was kept in osmium tetroxide at 37° C for varying periods, and the results were compared with Sudan black preparations. My preparations show that formaldehyde-saline material osmicated for 40 hours gives almost the same picture of the Golgi bodies as the Sudan black preparations. If the impregnation is further prolonged the lipid component appears thicker, and other artifacts also appear. My Sudan black preparations also show the duplex structure of the Golgi bodies.

In Kolatchev material the chromophobe substance (medulla) becomes greatly swollen, and the spheroids become flattened in the neurones of *Schistocerca*. Besides the bigger spheroids, there are also seen crescents and irregular rods as optical sections of flattened spheroids (Nath, 1944; Shafiq, 1953; Roque, 1954), or some of the rods are produced by joining up of the smaller spheroids (Shafiq, 1953). It can, therefore, be reasonably concluded

that osmication techniques do not produce artifacts *if used for an optimal period.*

The form of the Golgi bodies in the neurones of *Laccotrephes* is identical with the 'binary spheroids' of *Helix* (Thomas, 1947) and the Golgi elements of the vertebrate neurones described by Baker (1949). Generally there are one or two granules or a crescent attached to the medulla. A similar form of the Golgi body has been observed in this laboratory by Sud (1955, 1956) in the sperm-forming cells of the snake, *Natrix piscator*, and the tortoise, *Lissemys punctata*. He has also observed that the cortical component is sudanophil, osmiophil, and argentophil, whereas the medulla is stained by neutral red.

The medulla of the Golgi bodies of the neurones of insects (*Schistocerca* and *Laccotrephes*) is stained in life by neutral red. The medulla of the 'Golgi body' of Baker (1949), 'spheroids' of Thomas (1947), and 'lipochondria' of Shafiq (1953, 1954) and Roque (1954) are also stainable with neutral red.

There is no evidence that 'Golgi nets' exist in the neurones of insects. Monti (1915), as quoted by Shafiq (1953), described a Golgi network in arthropod nerve-cells; and Hosselet (1929) also described a network in insect neurones, formed by the hypertrophy of mitochondria. I believe in conformity with Beams and King (1932) that the 'nets' of Monti (1915) and Hosselet (1929) may be ascribed to the presence of neurofibrillae.

There is no evidence for the belief that these Golgi bodies are a complex of myelin figures (Palade and Claude, 1949, *a* and *b*).

I am greatly indebted to Professor Vishwa Nath for providing me with laboratory facilities, for suggesting the problem to me, and for valuable criticism in preparation of the manuscript. It is my pleasant duty to thank Shri B. L. Gupta, Technician to the Department, for taking the photomicrographs.

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A Histochemical Basis for Changes in Renal Tubular Function in Young Mice

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With one plate (fig. 2)

Summary

In the kidney of the adult mouse the tubular epithelium in the most proximal (P1) segment, corresponding roughly to the convoluted portion of the proximal tubule, shows strong alkaline phosphatase activity, and the brush border is moderately reactive to the periodic acid Schiff (PAS) method. In the more distal (P2) segment of the proximal tubule there is no alkaline phosphatase activity, and the PAS reaction of the brush border is intense. Examination of a large number of young mice has now revealed a distinct pattern in the development of this adult condition.

Differentiation of the two segments on the basis of the PAS reaction of the brush border becomes apparent on about the 15th post-natal day. The effect is the result of a decrease in the reactivity of the P1 segment. Differentiation of the two segments on the basis of alkaline phosphatase activity develops gradually between about the 22nd and 36th post-natal days. During this period the alkaline phosphatase activity disappears progressively from the proximal to the distal end of the P2 segment.

The administration of testosterone or estradiol to either sex accelerates the differentiation with respect to alkaline phosphatase activity. Castration of male mice retards the completion of this process.

It is suggested that morphological changes of this type may provide the basis for some of the functional differences between the kidneys of young and mature animals.

Introduction

WE have recently reported (Longley and Fisher, 1954) for the kidneys of a variety of animals a previously undescribed expression of segmental differentiation within the proximal tubule. This is based on the distribution of alkaline phosphatase activity and the PAS-reactive material in the brush border. In the mouse the effect is particularly well shown. In the pars convoluta and the upper part of the pars recta (P1 segment) intense alkaline phosphatase activity can be demonstrated, but none at all in the remainder of the pars recta (P2 segment); the intensity of the reaction of the PAS-positive material of the brush border varies in the opposite direction, a relatively feeble reaction being obtained in P1, but a brilliant one in P2.

These two materials serve purposes in the tubular epithelium which so far can only be guessed, but which appear to be significant. If so, it is clear that they are significant with respect to the intrinsic tubular mechanisms underlying kidney function.

These mechanisms have not been extensively invoked in accounting for the considerable changes in renal function which occur in young animals. We therefore believe it is a matter of interest that it has now been found that

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these materials undergo marked changes in distribution during the early lif of the mouse. It is the purpose of this paper to describe these changes, a well as some additional observations concerning the stimulus exciting them and to discuss their physiological implications.

Materials and Methods

Mice

As will be seen under the several experiments, mice of both sexes an various ages and genetic origins were used as the occasion required. Sinc some incidental interest attaches to our data with respect to genetic differenc in the mice, the various types used are characterized in this respect her C (BALB/c), C57 (C57BL/6), C58, DBA (DBA/2), A, and C3H are highl inbred strains described in Appendix 2 of the *Standard Nomenclature* (Carte and others, 1952). Mice designated NIH and CFW originated from whit Swiss and highly inbred CFW stock, and have been maintained at th National Institutes of Health in a somewhat inbred condition by non-selectiv brother-sister matings. GP (general purpose) refers to non-inbred whit Swiss mice.

Techniques

Kidneys were removed from mice under deep ether or chloroform anaes thesia and placed immediately in 65% alcohol at room temperature for fixation After 24 hours they were dehydrated in several changes of 95% and absolut alcohol, split sagitally with a sharp razor blade to facilitate the cutting of we oriented sections later, and cleared in petroleum ether. The tissue was rapidl imbedded in paraffin in the vacuum oven, and sections were cut at 5 μ. O adjacent sections alkaline phosphatase activity and PAS-positive material were demonstrated according to the procedures described by Lillie (1954).

Observations

Experiment 1. The variability of the segmental effect with alkaline phosphatas

This experiment, which was carried out shortly after we first observed th differential segmentation of the mouse proximal tubule summarized in ou introduction, was intended to show whether we had thereby discovered species characteristic, or merely a peculiarity of the female white Swiss mic with which we had been working. It is known that distinct differences ca occur between different strains of inbred mice; with regard to the structur of the kidney in particular, Dunn (1949) had already shown differences bot between strains and sexes. For this reason we took for study one male an one female each of C57, C58, DBA, A, C3H, CFW, NIH, and GP mice Unknown to us at the time was the fact that all these animals were weanling (23–30 days old), much younger than the mice in our own laboratory, whic are commonly kept for indefinite but extended periods.

The results with the PAS method were in all cases like those previously

escribed, but the distribution of alkaline phosphatase varied markedly. In ome cases P2 was negative as before, but in most some activity was demon-trated, ranging up to intensities indistinguishable from that in P1. In the ntermediate cases activity tended to be greatest in the distal end of P2.

The correlation between the degree of P2 activity and either sex or strain vas dubious; but we were unable to rule out either, particularly strain, as a etermining factor.

Experiment 2. The determining factor in variability of segmental effect

This experiment was intended not only to permit a definite conclusion vith respect to the possible significance of sex or strain, but also with respect o age. The last factor was suggested again by Dunn's study (1949) which mplies that age changes occur in the distribution of alkaline phosphatase in he kidneys of mice, but without making clear their nature. With this prompt-ng we also learned of the age difference between our own mice and those of Experiment 1.

The experiment was based on Dunn's, but in some strains the number of nice used represents an increase. Alkaline phosphatase in the kidneys of hree mice of each sex of strains A, C3H, C, DBA, and C57 was examined t three approximate ages: newborn, weanling (23–30 days), and young breeders (3–4 months).

The results obtained greatly clarified the situation. The kidneys of all new-born mice were alike with respect to alkaline phosphatase activity, but n weanlings we again encountered the variability seen in Experiment 1; and n mature animals, regardless of sex or strain, the enzyme activity was in all cases absent from the P2 segment. The segmentally differentiated distribution of this enzyme was thus shown to be a uniform characteristic of the species, but subject during maturation to some redistribution.

Experiment 3. The development of the segmentally differentiated state with respect to alkaline phosphatase and the PAS reaction of the brush border

To establish the details of the redistribution of alkaline phosphatase and possible similar changes, relative to age, in the PAS reaction, we studied series of C, C57, CFW, NIH, C3H, A, DBA, and GP mice. Animals were killed at 2-day intervals during the week with a 3-day interval over week-ends. New-born mice having already been examined, the first were killed at the age of 3 days; the last were killed at 54 days. Since the observations of our two previous experiments showed no striking differences between sexes in the maturation process, mice of either sex were taken at random in these series.

The staining achieved in the alkaline phosphatase reaction of the P2 segment varied from none to very black, and we found it possible to rate degrees of staining on a 0–4 scale with a high degree of consistency between observations on the same material made independently by each of us. The ratings assigned to individual mice of each strain have been plotted against age in the graphs of

fig. 1. The changes observed in the PAS reaction, though equally definite do not achieve sufficient tinctorial extremes to be estimated in this way. Th following sequence of events was observed in every strain.

1–10 days.

In mice of this age the organization of the kidney is quite different from that of the adult, which has been figured by Dunn (1949). Hamburger (1890) has described the development of the mouse kidney at length, and only the salient features need be noted here. Directly under the capsule is the neogeni

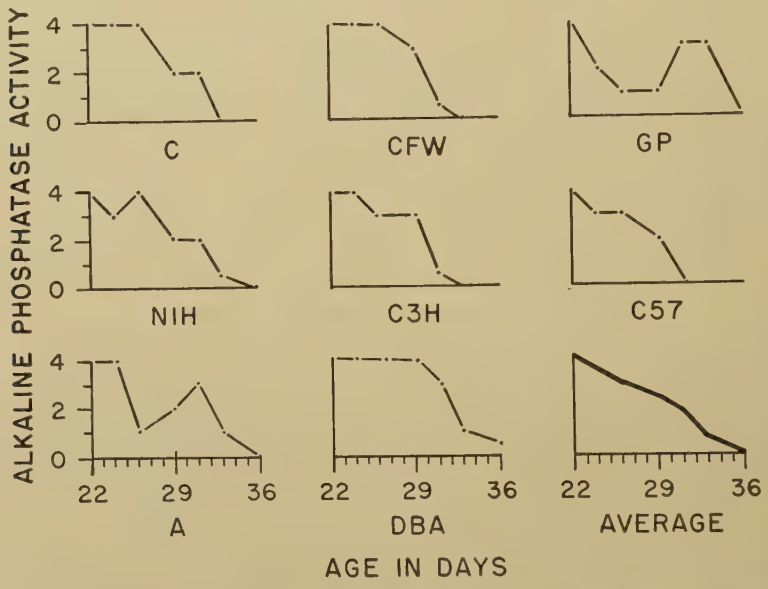

FIG. 1. Normal regression of alkaline phosphatase activity in P2 segment of mouse kidney tubule.

zone, in which new glomeruli and tubules are still differentiating. Below this lies a zone of convoluted tubules and mature glomeruli. These first two zones constitute in the adult the outer zone of the cortex. Beneath these lies a cortico-medullary zone possessing a conspicuous stroma of fusiform cells which show a marked tendency to transverse orientation. This stroma is penetrated deeply by the straight terminal segments of the proximal tubules of the second zone and traversed conspicuously by isolated collecting tubules and straight segments of the distal tubule. This zone differentiates to form in the adult the inner and outer stripes of the outer zone of the medulla. These three zones are well shown in fig. 2, A. The remainder of the medulla need not concern us here.

The proximal tubules of the second zone and their continuation into the third zone have distinct brush borders which stain brilliantly with the PAS reaction (fig. 2, A). Alkaline phosphatase activity is found to correspond well

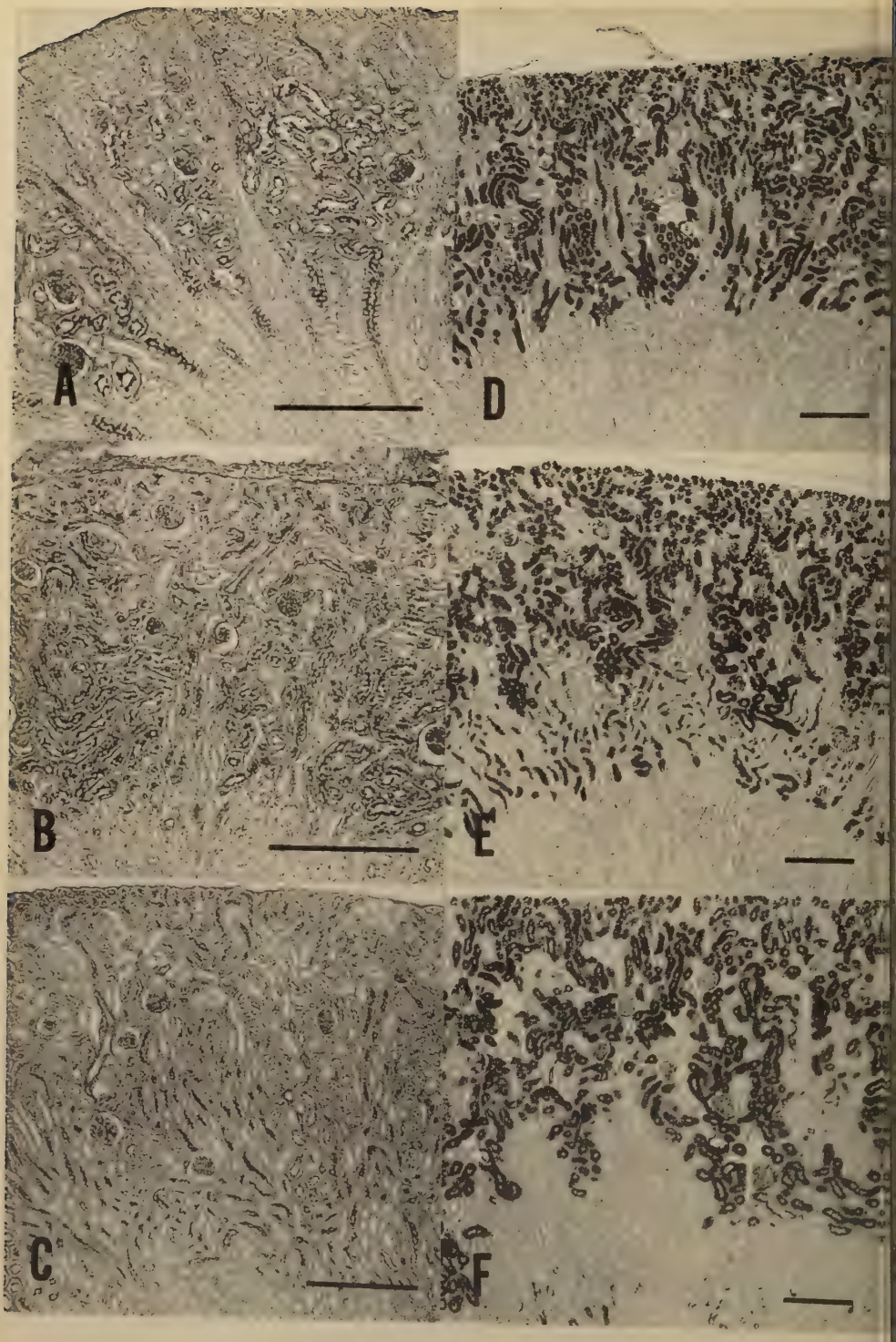

FIG. 2

with this in distribution, though it cannot always be demonstrated to the distal limit of the proximal tubules. During this period more and more of the recognizable tubules of the outer neogenic zone become similarly reactive and its existence as a well-defined entity is gradually ended. The stromal tissue of the cortico-medullary zone also largely disappears during this time, but its fate is not clear.

10–15 days.

During this period growth supersedes differentiation as the conspicuous feature of development, though diminishing numbers of apparently immature glomeruli can still be found. At the beginning of the period the PAS reaction of the brush border of the proximal tubule is brilliant and uniform throughout (fig. 2, B), but between the 12th and 15th day the intensity of the reaction in the true cortex gradually fades, thus presenting at the end of the period in this respect essentially the same segmentally differentiated picture seen in all older mice (fig. 2, C). This is not accompanied by any change in the distribution of alkaline phosphatase activity.

15–22 days.

During this week the distribution of alkaline phosphatase activity and PAS reaction shows no further change.

23–33 days.

Between the 22nd and 24th day usually, but in some mice considerably later, the alkaline phosphatase activity of the distal (P2) segment of the proximal tubule begins to diminish. The termination of the process is also variable, but it is almost always completed by the 36th day. In two of our animals, however, it was incomplete at 50 days.

Rather than proceeding as a uniform reduction, it is characteristic that the enzyme disappears from the proximal end of the segment first and gradually

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FIG. 2 (plate). The photomicrographs represent sections of the kidney of the mouse. A-C, PAS reaction; D-F, alkaline phosphatase (cobalt) method, counterstained with eosin. The scale-marks in each case represent 200 μ.

A, 5 days old. The relatively undifferentiated proximal tubules lying in the outer (neogenic) layer of the cortex have not yet acquired a reactive brush border.

B, 12 days old. Proximal tubules at all levels have a uniformly and strongly reactive brush border.

C, 15 days old. The P1 and P2 segments of the proximal tubules are clearly distinguished by the marked difference in intensity with which their brush borders are stained. The difference appears to arise through a decline in the reactivity of the brush border of the P1 segment.

D, 22 days old. Proximal tubules in the cortex and outer zone of the medulla show no discernible variation in phosphatase activity.

E, 29 days old. The distinction between the outer stripe of the outer zone of the medulla and the cortex, composed of P2 and P1 segments (so far as the proximal tubule is concerned), becomes visible owing to the loss of activity in the inner zone, most conspicuously along the line of junction of the two zones.

F, 35 days old. The phosphatase has retreated down the P2 segments and is now detectable only at their distal extremities. These sites are seen scattered just above the lower edge of the figure.

recedes down the tubule, so that the last level to lose it is exactly at the junction of the proximal tubule with the thin limb of Henle's loop. Fig. 2 D–F, illustrates this process. With the complete disappearance of phosphatase activity in this segment, so far as these methods show, the kidney is fully matured.

Experiment 4. The effect of administered testosterone and estradiol

Kochakian (1948) has shown that sex hormones have some relation not only to kidney growth, but also to alkaline phosphatase levels in the kidney. That the changes we have described in the kidney of the mouse occur over a period of presumably increasing secretion of sex hormones suggests that these hormones may be exercising a regulatory effect here. The results of the following experiment are compatible with this hypothesis.

At 6 days of age, 40 of 100 GP white Swiss mice received intraperitoneally 1 mg of estradiol suspended in water, 40 had a small drop of a 100 mg/ml solution of testosterone propionate in oil (Oreton Shering) rubbed on the skin of the back, and 20 were left untreated as controls. Those receiving the estradiol received no further treatment during the experiment, but those receiving testosterone had the original treatment repeated every two days. Mice from each group were killed on the 10th day after birth and daily after that up to the 27th day. Sexes were recorded at autopsy.

The effect of these treatments on the alkaline phosphatase of the P2 segment were estimated as before, and the results are expressed graphically in figs. 3 and 4. The differentiation of the P1 and P2 segments with respect to PAS reaction showed qualitatively an acceleration also.

Experiment 5. The effects of castration

To test further the involvement of the sex hormones in the induction of the changes observed, 8 GP males were castrated at age 15 days. Four mice survived until age 56 days, at which time they were killed. Each of these animals showed slight to moderate persistent alkaline phosphatase activity scattered irregularly throughout the P2 segments.

Discussion

Physiological significance.

Very considerable functional differences exist between the kidneys of young and mature animals (McCance, 1948; Smith, 1951, pp. 492 ff.). On theoretical grounds it can be assumed that these arise either from changes in the general structure and organization of the kidney, or from changes in the functional capacities of the tubular epithelium itself. Morphological studies of the kidney during post-natal growth have given ample evidence of changes of the first sort—changes in the relative bulk of the various segments of the tubules, thinning of the membranes of the glomerular tufts, and so forth—but changes in the character of the tubular epithelium which might reflect changes in intrinsic tubular function have been largely wanting.

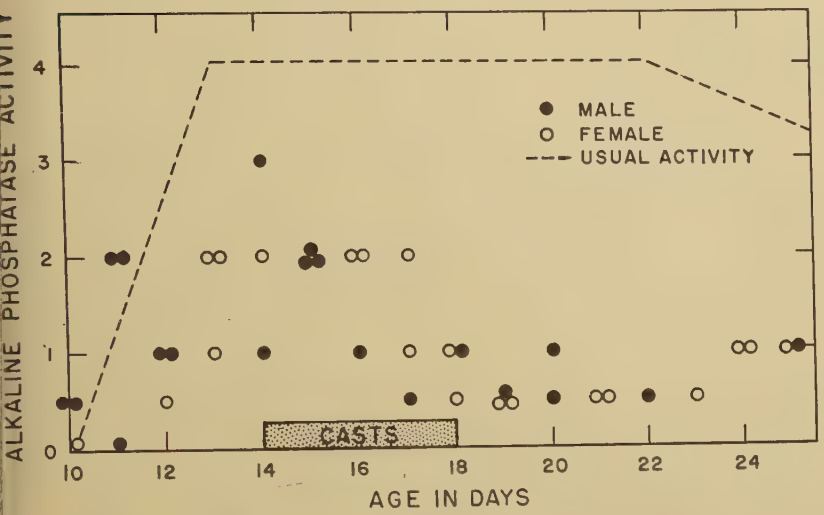

FIG. 3. Induced regression of alkaline phosphatase activity in P2 segment after cutaneous application of about 2 mg testosterone on even numbered days from age 6 days (GP mice).

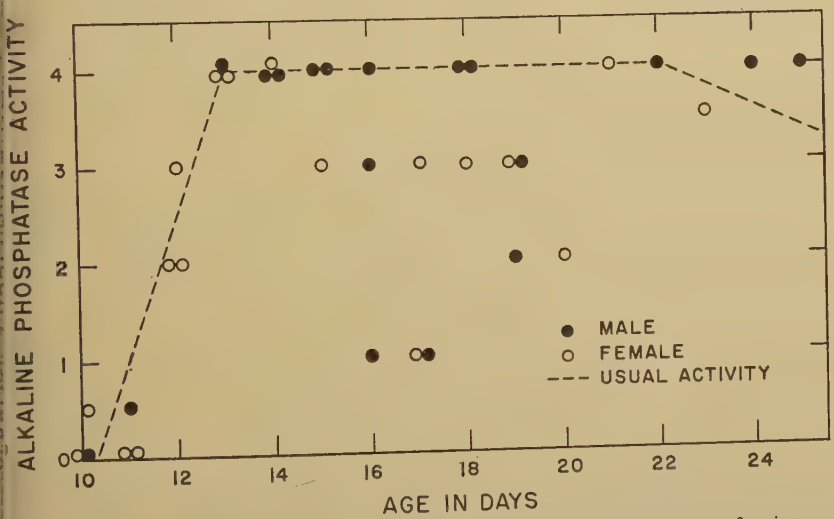

FIG. 4. Induced regression of alkaline phosphatase activity in P2 segment after intraperitoneal administration of 1 mg of estradiol at age 6 days (GP mice).

From the physiological point of view these results are satisfactory. Smith (pp. 503 ff.) has shown that the known changes in the organization of the kidneys of young animals form a reasonable basis for explaining all the main features of renal functional maturation. There are, nevertheless, hints that intrinsic tubular mechanisms are involved. Galán and his colleagues (1947, 1949) have reported that in children 2 to 11 years old the maximal tubular resorption of glucose greatly exceeds adult levels. This observation is in marked contrast with a number of other proximal tubular functions, which at this age might be expected to have reached adult levels, but hardly to exceed them. The implication here of the independent variation of functions believed to reside in the same part of the nephron is difficult to account for on the basis of glomerular-tubular balance (Smith, p. 513). On the morphological side Baxter and Yoffey (1948) have observed that in new-born rats only a few proximal tubules possess brush borders and have the ability to store trypan blue in the tubular epithelium, and that the remaining tubules gradually acquire these characteristics together over the first 30 days of life. In neither of these cases, however, is the suggestion entirely convincing. The observations of Galán and colleagues involve relatively few individuals, and therefore leave some doubt as to whether their results are representative. In regard to the findings of Baxter and Yoffey there is certainly indicated a post-natal change of function so far as the individual tubules affected are concerned, but for the kidney as a whole the implication is quantitative rather than qualitative. From the point of view of glomerular-tubular balance this differs little from any other process changing the effective mass of proximal tubular tissue.

With respect to the present observations, the initial development of alkaline phosphatase activity and the PAS reaction in each tubule is a process analogous to that observed by Baxter and Yoffey, in that the most mature tubules present at birth already show these characteristics. The class of functional effects with which they are compatible is therefore also the same. But it is further apparent that the subsequent changes must be of quite different significance. In differentiating the two segments of the proximal tubule they demonstrate in two separate instances that tubular characteristics, and hence presumably tubular functions, can vary independently after birth. Further, from the fact that none of the proximal tubule retains in the mature mouse the same combination of characteristics as any part of the tubule present at birth, presumably no part of it functions in the same way. There is therefore a strong presumption, though still less than certainty, that the process observed contributes to detectable differences in the urine of young and mature mice. If it can be accepted that changes of intrinsic tubular function are probably a factor in renal maturation in the mouse, it would seem likely that the same process might play a part in renal maturation in other species. It would certainly be one possible explanation of the findings of Galán and his colleagues, and it could indeed account for other facts of functional maturation in the kidney.

Hormonal influence

The responses obtained through the administration of testosterone and estradiol are striking, but still leave doubt as to whether these hormones are the natural stimuli for this aspect of maturation. In the absence of suitable determinations the assumption that their titre is increasing over the interval in question is speculative. Further, although the effects of castration are distinct, the arrest produced is by no means complete. It seems possible from this that steroids from sources other than the gonads may compensate the experimental deficiency to some extent. No conclusions are justified on the relative effectiveness of the two hormones on the basis of the data presented, owing to differences in the mode of administration.

Strain differences

In addition to characteristic strain differences in the age at which regression of the P2 alkaline phosphatase begins (these appear to be reflected in some of Dunn's (1949) observations), there is suggested by the curves of recession for A and GP mice in fig. 1 a somewhat modified pattern of maturation characterized by periods of resurgence before the final disappearance of the enzyme. Studies of much larger numbers of mice of these types, due to a wide range of individual variation and, no doubt, the crudity of the estimation, have not resolved the question either way.

This paper was presented at the 1955 meeting of the Histochemical Society. Some further informal discussion is reported in the Proceedings (Longley and Fisher, 1955).

We are greatly indebted to Mrs. Helen J. Burtner for technical assistance in this work.

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A Microscopical Finder-slide

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SUMMARY

The finder-slide is produced by photographic reduction of a typescript chart. By its se, recorded objects can be re-located on different microscopes.

THE problem of locating objects of interest on a microscope slide, so that they may be found again for subsequent study, has always been of great mportance. Several suggestions have been put forward in order to achieve his end, e.g. finder-slides and verniers on a mechanical stage. A finder-slide onsists of a reference grid, which when substituted in place of the object slide, nables a reading to be taken, so allowing the position of the slide to be uplicated at any time. Such a device was published by Maltwood in 1858. At rst he used coloured lines drawn out on paper which was then gummed to the lide, but soon this was abandoned in favour of a series of squares, one-fiftieth f an inch across, each containing two reference numbers enabling the square b be located by giving its 'latitude' and 'longitude'.

The squares forming his original chart were drawn out by hand on a sheet f paper 10 inches square, which was photographed to give a negative 1 inch quare. From this a positive was printed, and after grinding the edges of the lide, a cover glass was cemented over the photographic emulsion. Later, Maltwood used smaller squares and instituted the idea of printing many nder-slides from one accurately ground negative.

These Maltwood finders have been unobtainable for many years. An exmple now in the possession of Dr. J. R. Baker led us to consideration of the ossibility of producing a similar finder-slide by modern methods. This was chieved as follows. A table was typed on several sheets of white paper which ere later glued together to form one large chart, 2 ft 6 in. by 1 ft 8 in., ivided into 100 × 60 squares, each square containing two numbers indicating latitude' and 'longitude'. The top row was numbered 1, the second 2, and so n down the chart to row 60. Horizontal columns were numbered 1–100 from eft to right. When complete, the chart was photographed to give a quarterlate negative on Ilford N 40 Process plate. Great care was needed in arrangng the lighting in order to ensure that the large chart was evenly illuminated. The final arrangement consisted of four 40-watt 'Silverlight' bulbs, one at ach corner of the paper, the evenness of the illumination being checked by se of a Weston meter. Exposure was 8 seconds at an aperture of f. 12, and the eriod of development in Ilford caustic hydroquinone (I.D. 13) was 2 minutes t 20° C. When dry, the negative was masked with lantern-slide binding-strip o produce a neat print. Printing was by projection, the final size of the image eing $1\frac{3}{4}$ by $1\frac{1}{4}$ in. The prints were made on lantern slides (Ilford special antern plate, normal grade) which had been cut in half. Care was taken to nsure that the intended lower edge and left-hand side of the finder were

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always formed from the original edges of the lantern plate. When printing, th edge of the chart was brought very close to the lower edge of the lantern plat in order that there should always be a portion of the finished chart in the fiel of view of the microscope, wherever the detail might be on the object slide. I order to obtain interchangeability of finders, it was necessary, during printing to see that each cut lantern-plate was well pushed up to the edges of the print ing mask. The period of development was 2 minutes at 20° C in I.D. 1 After fixing, washing, and drying in the usual manner, the photograph of th chart was mounted in DPX under a number 1 coverglass. No heat was applie during the drying, as this would probably have caused some melting of th gelatine emulsion base, with consequent distortion of the lines and figures. I might be possible to use gentle heat to help drying if, before mounting, th slides were passed through a weak formalin bath in order to harden th gelatine. When dry, the superfluous mountant and emulsion were removed b scraping with a razor-blade and the finder finished with a 'ringing' of blac enamel.

There are three main differences between our technique and that used b Maltwood. (1) A typewriter was used to prepare the chart, in order to mini mize the labour of writing out some thousands of figures. (2) Photographi reduction of this to the final size took place in two stages. (3) Printing was b projection. The second and third items help to minimize the loss of definitio that may occur if single-stage reduction is followed by contact printing.

In one batch of twenty finders produced at the same time, the degree c interchangeability is good, the maximum deviation being only one squar throughout the batch. Between the two best finders the deviation is only one quarter of a square. If extra accuracy with respect to interchangeability i desired, the edges of the lantern plates may be ground before printing.

The method of use is very simple. The object to be logged is placed in th centre of the microscope field. The slide is removed and replaced by the finder without movement of the stage of the microscope. The finder is focused wit the 16 mm objective and the number of the square occupying the centre of th field is noted. Readings may also be taken with the 4-mm objective, one squar then occupying almost the whole field. To locate the object at any future time this procedure is reversed. It must be noted that the finder-slide can only b used on a microscope equipped with some form of locating device, so that th slides can always be replaced in the same position relative to the stage. Should a greater degree of localization be necessary, Maltwood's original system ma be used. He gave the square reference in the usual manner, and then marke the position of the object within the square by means of a cross on a smal drawing of a square.

One of us (W. G.) is indebted to the Medical Research Council for financia support.

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Further Observations on the Histochemistry of Fat Absorption in the Small Intestine of the Rat

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With one plate (fig. 1)

Summary

Studies have been made of the small intestine of rats previously fed with triolein. previous description of the location of the lipid and phospholipid has been con- rmed by using Sudan black and the acid haematein test. In addition histochemical sts have been applied to detect neutral fat and fatty acid. The lipid in the free border the epithelial cells of the mucous membrane consisted of fatty acid and probably eutral fat, which would support the belief that when neutral fat is absorbed some of is unhydrolysed. Additional evidence for this is provided by the fact that neutral fat ould also be detected between the lateral parts of the cells. Occasionally fatty acid as also detectable in this location. Variable proportions of neutral fat, fatty acid, and hospholipid were to be found inside the epithelial cells and it appeared possible that here was a direct relationship between the amounts of neutral fat and phospholipid resent.

Introduction

N a previous publication (Hewitt, 1954) a description was given of the location of the lipid in the small intestine of rats previously fed with triolein. o details are given of the identity of any of the materials composing the lipid ther than the phospholipid. To complete this aspect of the work the experi- ents were repeated, with slight modification, and histochemical tests for eutral fat and fatty acid were also applied.

Material and Methods

Rats of both sexes, from the animal-house stock, weighing from 177 to oo g were used. They were starved for 48 hours but allowed free access to ater, and during this period they were kept separately in large mesh cages. Each animal was fed on 225 mg (0·18 ml) of triolein by gastric intubation, s in previous experiments. After $2\frac{3}{4}$ hours it was killed by a blow on the head. Chloroform was used in the previous experiments and was criticized on the round that it might have caused intestinal contraction.

Pieces of intestine, each 1 cm long, were removed at intervals of 20 cm, ommencing 5 cm from the pylorus. Each piece was washed through with Baker's (1944) formaldehyde-calcium and then fixed in this for 6 hours. In wo experiments each piece was slit open along its length before fixation, and his led to improved results.

After fixation for 6 hours the tissues were subjected to the acid haematein

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test for phospholipid (Baker, 1946) to the stage when frozen sections 10μ in thickness were cut. Some sections were then treated with a saturated solution of Sudan black in 70% alcohol for the detection of lipid, whilst others were subjected to the Nile blue technique of Cain (1947 and 1948) and Mallory's (1938) modification of Fischler's (1904) technique for fatty acids. The acid haematein test was completed on the remaining sections. Whilst not absolutely specific, the Fischler technique was probably quite reliable for the detection of fatty acid when this was known to be a likely constituent of lipid already detected in the same location by Sudan black. The Nile blue technique of Cain gave a pink colour with neutral fat, and with acidic lipid it gave a blue reaction. The majority of the acidic lipid consisted of phospholipid and fatty acid and the relative amount of each was determined by comparing the results of the Nile blue reaction, the Fischler technique, and the acid haematein test. It has been found advisable to examine the Nile blue preparations as soon as completed because the blue reaction given by acid lipid developed more slowly than the pink reaction of neutral fat. The latter was, therefore, more in evidence in newly prepared sections. In a few hours, when the blue reaction had completely developed, it tended either to obscure the pink material altogether or mix with it and produce a violet colour. Furthermore, the blue colour tended to diffuse throughout the whole section and colour all the lipid present including the neutral fat, thus giving a false localization. The cause of this was not decided, but it may have been a peculiarity of the technique or due to the behaviour of triolein or oleic acid. Attempts to overcome it by varying the acetic acid differentiation, prolonging the final washing, or using glycerine jelly or Farrants's medium as a mountant were unsuccessful. In view of these facts it was considered to be more advantageous to perform the Nile blue reaction when all the other reactions had been completed, so that a better comparison could be effected and the acidic lipid more accurately located.

Results

The Sudan black preparations confirmed the previous description (Hewitt, 1954) of the location of the lipid within, between, and in the striated border of the epithelial cells. In addition, in these Sudan black preparations, the core of the villus contained a variable quantity of lipid particles with an occasional conglomerate mass of lipid in or near the position of the lacteal. This lipid contained neutral fat and fatty acid in varying proportions.

Contrary to the findings of Baker (1951) the spindle-shaped lipid particles in the free border reacted with Nile blue and also with the Fischler technique. Some of the material in the free border was undoubtedly fatty acid, but the presence of neutral fat could not be excluded. The pink colour could certainly be seen in the free border, but whether or not it coincided only with the spindle-shaped particles was impossible to decide owing to the lack of adequate contrast with this reaction. However, the fact that the pink colour could be detected here at all suggested that neutral fat was probably present.

20 μ

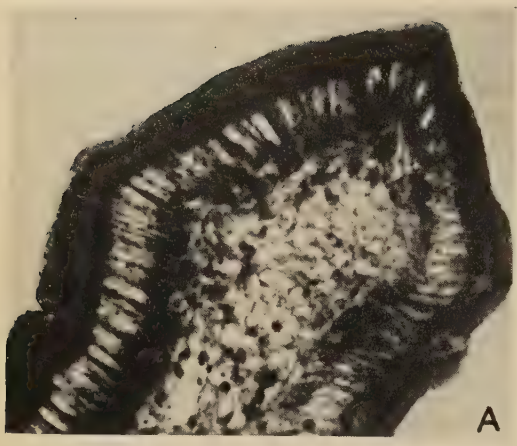

FIG. 1

W. HEWITT — PLATE I

Lipid particles in the intercellular regions between the lateral parts of the pithelial cells usually consisted of neutral fat, but more rarely fatty acid articles could be detected in this region. These streams of intercellular lipid oined those at the sides of the nuclei, which here consisted of phospholipid, eutral fat, and fatty acid in varying proportions. The pink material revealed y Nile blue in this intercellular lipid usually had a hyaline rather than the ustomary particulate appearance. However, this same hyaline appearance was lso to be seen in the central lacteal, where neutral fat was to be expected, and his supported the belief that this pink-reacting intercellular material was n all probability neutral fat.

The intracellular lipid in the outer part of the cell on the lumen side of the ucleus and in and around the cell below the nucleus consisted of phospho- ipid, neutral fat, and fatty acid in varying proportions. The location of the hospholipid in the cell has already been described (Hewitt, 1954). In addition o this a rough estimate was made of the quantity present, for there appeared o be a direct relationship between the amounts of phospholipid and of eutral fat present. This is illustrated in fig. 1, which shows sections of a villus where neutral fat is more abundant in the epithelial cells near the base, which lso contain more phospholipid, than in those near the tip of the same villus where phospholipid is almost absent. This may, of course, have merely been he result of unmasking the neutral fat, which had come from the intestinal umen, by the incorporation of the blue-reacting fatty acid in the phospho- ipid formed (Sinclair, 1929, 1936; Artom and Peretti, 1935; Artom, Sarzana, Perrier, Santangelo, and Segré, 1937; and others). On the other hand, it would be equally true that the blue-reacting phospholipid formed would also probably have obscured the neutral fat.

The possible locations and chemical composition of the lipid in and around an intestinal epithelial cell and core of a villus are depicted in the diagram (fig. 2).

FIG. 1 (plate). Photomicrographs of sections of villi, 10μ in thickness, from the same segment of the small intestine of a rat, 2¾ hours after feeding with triolein. All the preparations are typical of the appearances throughout the segment.

A, Sudan black preparation. Tip of a villus showing the location of the lipid particles, which can be seen in the epithelial cells around their nuclei. The majority of this lipid consists of fatty acid and only a very small amount of neutral fat and phospholipid.

B, Sudan black preparation. Base of the same villus as A. The disposition of the lipid is similar to that in A but in addition a few particles can be seen scattered in the outer part of the cells and occasionally between them. The lipid here is almost entirely composed of neutral fat and phospholipid. Only a small amount of the lipid in the region of the Golgi apparatus consists of fatty acid.

C, acid haematein preparation. Tip of a villus showing the almost complete absence of phospholipid from the cells except for a small amount below the nuclei in some cells.

D, acid haematein preparation. This is from the base of the same villus as C. In complete contrast with C the epithelial cells contain a high concentration of phospholipid in both their outer parts and in their bases below the nuclei. This increased concentration of phospholipid appears to be associated with the amount of neutral fat present in B. Compare with C, where neutral fat is almost absent.

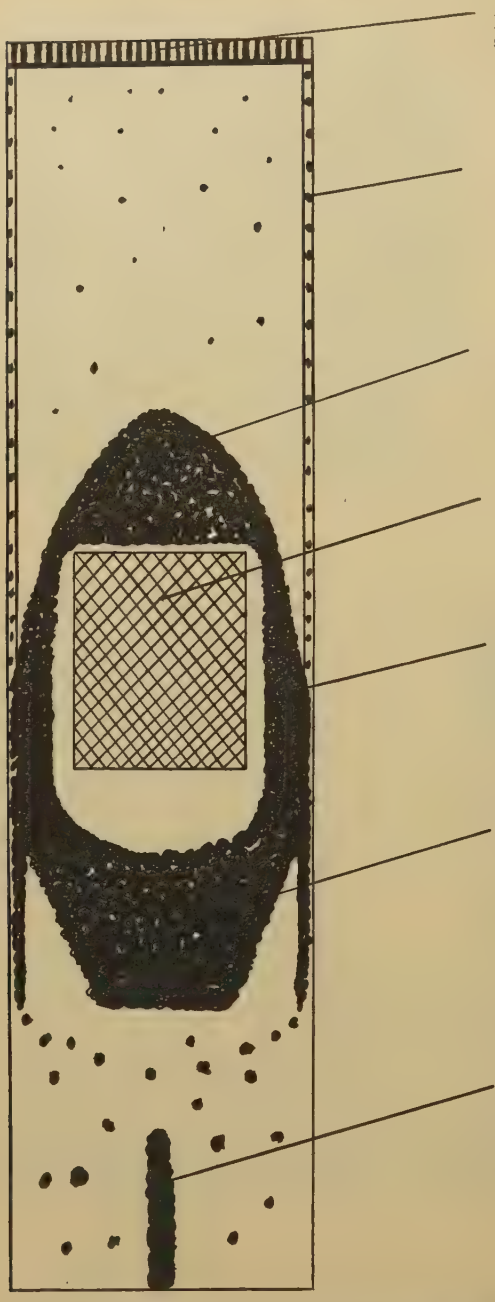

FIG. 2. Diagram depicting the possible locations of the lipid, with its chemical composition, in an intestinal cell and core of a villus of a rat, during the absorption of triolein.

Conclusions

Two hypotheses have been advanced to explain the chemical mechanism involved in the absorption of a triglyceride fat from the small intestine. The essential difference between these concerns the degree of hydrolysis undergone by the fat, before its absorption through the intestinal mucosa. In these experiments the presence of a positive reaction for neutral fat in the striated border lent support for the belief that during the absorption of the triglyceride some of it was in an unhydrolysed condition. The neutral fat between the outer parts of the cells may have been derived from that within the cells but it is much more likely that it too was derived from the intestinal lumen. Neutral fat can always be recovered from the thoracic duct during the absorption of a triglyceride, and if this has been totally hydrolysed before its absorption, then at some stage neutral fat must have been synthesized. It has been suggested that the phospholipid which is formed in the epithelial cell during the absorption of triglyceride takes part in this synthesis of neutral fat. Although the results presented here did not substantiate this hypothesis they suggested the possibility that a link existed between the phospholipid in the cell and the amount of neutral fat which was present. It must be emphasized, however, that with the technique employed here it was impossible to obtain a quantitative estimate in support of this relationship. Further studies will be necessary before the function of the phospholipid inside the intestinal cell is understood.

I wish to thank Professor R. G. Harrison of the Department of Anatomy, University of Liverpool, where this investigation was commenced, and Professor D. V. Davies of this Department, where it has been continued. I also wish to thank Mr. J. S. Fenton for his technical assistance.

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Distribution of Iron in *Daphnia* in Relation to Haemoglobin Synthesis and Breakdown

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Summary

Daphnia synthesizes haemoglobin in poorly aerated water and loses it in well-aerated water. A histochemical study has been made of iron in the tissues of parthenogenetic female *D. magna* when gaining or losing haemoglobin, and with a steady content of the respiratory pigment.

In animals gaining haemoglobin, loosely-bound iron was found especially in the gut-wall, fat-cells, and ovaries. The iron in the gut-wall was probably being absorbed into the body, and that in fat-cells and ovary being incorporated into newly synthesized haemoglobin.

In animals losing haemoglobin, loosely-bound iron was found especially in the walls of the gut caeca, in the fat-cells, and in the excretory shell-glands. Several lines of evidence indicate that iron was being excreted by the shell-glands. It was probably also being excreted through the gut caeca. In the fat-cells haemoglobin can accumulate during haemoglobin loss and it is probably broken down here with the deposition of iron. This iron is subsequently excreted.

Introduction

THE pond species of *Daphnia* possess haemoglobin in the blood-plasma, but unlike most animals with this blood-pigment they vary greatly as regards the concentration of haemoglobin present in a single individual at different times. The main cause of variation is the quantity of oxygen dissolved in the water in which *Daphnia* swims: in poorly aerated water the animals are red, in well-aerated water they have no pink colour (Fox, 1948). The change from one condition to the other may be rapid and the quantity of haemoglobin synthesized or lost can be very considerable. In addition to paucity of oxygen, iron in the water augments the synthesis of haemoglobin in *Daphnia* and a combination of the two factors can increase the haemoglobin concentration about tenfold in a fortnight (Fox and Phear, 1953, fig. 2). This remarkably rapid gain and loss of haemoglobin naturally raises the question of the sites of synthesis and excretion of the pigment, and of the nature of its breakdown products, if any.

We know that one route by which haemoglobin is lost from the body of female *Daphnia* is through the parthenogenetic eggs. These are laid into the brood-pouch just after each moult, which recurs in *D. obtusa* at intervals of 52 hours when the temperature is 22° C. The eggs contain haemoglobin derived from the mother's blood (Dresel, 1948), and in supplying this the blood loses to the ovary up to one-third of its haemoglobin, which is regenerated in the course of the next instar. When red *Daphnia* passes from poorly into well-aerated water, the haemoglobin that went from the blood into the eggs is no longer regenerated, and after four moults about 80% of it will have been lost

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to the eggs (Green, 1956). Clearly the eggs are an important route for haemoglobin loss in parthenogenetic females, but fertilized eggs contain no haemoglobin (Fox, 1948), and males also lose haemoglobin, indeed more rapidly than females (Green, 1955*a*). In parthenogenetic females, too, there may be another route of haemoglobin loss than through the eggs.

Occasionally an individual *Daphnia* is found in a population of red or pink animals with one of its two excretory organs, the maxillary or so-called shell-glands, bright red in colour. The microspectroscope shows this colour to be that of oxyhaemoglobin. In such animals the blood is usually pale. This is evidently a pathological condition in which haemoglobin has drained from the blood into the excretory organ, but it suggests that haemoglobin may perhaps normally be excreted as such by that route (Fox, 1948). It has, however, been impossible to detect haemoglobin in aerated water containing red *Daphnia* in the process of becoming pale, even when they are crowded.

In the gut lumen of *Daphnia* a protohaemochromogen is present, named daphniarubin (Fox, 1948). This could be derived from the haemoglobin of the blood. Daphniarubin has been studied in detail by Phear (1955), who was unable to decide to what extent this haemochromogen in the gut is derived from haemoglobin, but who showed that in the course of embryonic development haemoglobin undoubtedly changes into daphniarubin. As development proceeds in the brood-pouch, haemoglobin progressively decreases in the embryos while daphniarubin correspondingly increases.

In mammals the haemoglobin of the blood is continuously destroyed as the old red cells break down, and it is continuously synthesized as new red cells are formed in the bone-marrow. In the destruction iron is removed from the haem, the remainder of which is then excreted as bile pigment. It is thus natural to expect that when the haemoglobin content of *Daphnia* decreases, a bile pigment will appear, but none has been detected. H. Munro Fox (personal communication) tried in vain to find a bile pigment by chemical extraction, using large quantities of *D. magna*, and I have not succeeded in showing a bile pigment histologically in *D. magna* which was losing haemoglobin, by using the iodine method of Stein (1935). It may be that when red *Daphnia* becomes pale, so much haemoglobin passes into the eggs and so little is broken down that the bile pigment is too dilute to be detected. The haem of broken-down haemoglobin might, however, appear as porphyrin after removal of the iron. But no red fluorescence could be found either in *Daphnia* spp. themselves or in extracts made in glacial acetic acid and taken to ether and then to hydrochloric acid of various strengths (H. M. F., personal communication). This suggests that the haem may be broken down to simpler compounds than bile pigments or porphyrins.

If in red *Daphnia* which is becoming pale some of the haemoglobin is broken down, in addition to that which passes as such into the eggs or is perhaps converted to daphniarubin, then iron must be set free, and the present investigation was undertaken with the object of seeking such iron histologically. A preliminary account of part of the work has been published (Smaridge,

954). In addition, by studying the location of free iron in the tissues while *Daphnia* is gaining haemoglobin, it was hoped that light would be thrown on he site of synthesis.

Culture Methods

Mature parthenogenetic females of *Daphnia magna* Straus were used. The ulture methods were those described by Fox, Gilchrist, and Phear (1951), which are briefly as follows. In order to cause red animals to become pale, or to maintain colourless populations (i.e. lacking the pink colour of dilute haemoglobin), *Daphnia* was kept in open troughs. To induce haemoglobin ynthesis in colourless animals, or to maintain red populations, conical flasks were used. In these the oxygen content of the water was initially reduced by bubbling nitrogen through it, and then the respiration of the animals kept the oxygen content low. If it became too low, warning would be given by the animals swimming near the surface, and then the surface was slightly increased by removing water. *Chlorella*, cultivated on agar slopes, was used as food, its concentration being controlled by a photometer. The cultures were kept in the dark. Adequate feeding was judged by the average number of eggs or embryos n the brood-pouch, which was rarely less than a dozen.

While *Chlorella* seems an adequate diet as judged by egg number, more haemoglobin is synthesized when extra iron is added (Fox and Phear, 1953). It was found that if all cultures were given small amounts of additional iron, the histological distribution of iron could be more easily studied. This iron was given by adding 1·0 ml of a stable ferrous ammonium sulphate solution, containing 0·1 mg iron, daily to every 100 ml culture water. When larger quantities were added, iron was found adsorbed on the exoskeleton, especially on the thoracic limbs and antennae. Since this might have invalidated the results, the added quantity of iron was kept low. The culture water was changed every 5 days, so that there was never more than 0·5 mg. iron in 100 ml water.

Distribution of Non-Haem Iron in the Tissues

As regards ease of detection, iron exists in tissues in three forms. (1) As 'loosely-bound' iron it can be revealed by simple chemical tests, such as Prussian blue, without previous treatment. (2) As 'firmly-bound' iron it must be treated with acid alcohol before the chemical tests are applied. (3) The iron of haem compounds, for example of haemoglobin and the cytochromes, is only revealed by more drastic methods, such as treatment with hydrogen peroxide, or microincineration.

The distribution of non-haem iron, (1) and (2), in the tissues of *Daphnia* was studied by using Turnbull's blue and Prussian blue histochemically to show the presence respectively of ferrous and ferric iron (Bunting, 1949). For loosely-bound iron (1), animals were fixed for 24 hours in 10% formalin buffered at neutrality, and then washed in two changes of 70% alcohol for 15 minutes. Staining took 30 minutes in freshly mixed 2% aqueous potassium

ferricyanide (for Turnbull's blue) or ferrocyanide (for Prussian blue) with an equal quantity of 2% hydrochloric acid. The material was then washed in distilled water, dehydrated, and mounted in balsam. The colours were studied at once, so that subsequent fading did not matter. For firmly-bound iron (2) animals were fixed as before and then left in 95% alcohol containing 3% concentrated nitric acid for 24 hours at 35° C. This released ('unmasked') the iron, so that it could react with the Turnbull's or Prussian blue reagents. After the treatment with acid alcohol most of the iron is ferric.

Although formaldehyde fixation of *Daphnia* resulted in poor cytological detail, it gave the most consistent results for iron staining. Bouin's fluid, although recommended by Lison (1936), Wigglesworth (1943), and Gomori (1951), was found to be useless for iron in *Daphnia*. Alcohol fixation resulted in diffusion of Prussian blue, seen during and after staining. Formaldehyde, however, has one disadvantage: after its use most of the iron is in the ferric state, whereas alcoholic fixation showed that some of this may originally have been ferrous. Diffusion of iron occurs, too, during the unmasking of firmly-bound iron by acid alcohol, and for this reason the results with loosely-bound iron are alone given in detail here. With loosely bound iron and formaldehyde fixation, no blue colour was seen to leave the specimens, either during staining or dehydration.

Care was taken to avoid having iron in the reagents or instruments, which might have been adsorbed by tissues. Glass-distilled water, iron-free alcohol and glass instruments were used, while acids were tested for contamination. As nuclei rarely showed a blue colour in the loosely-bound iron technique it is concluded that the difficulty was overcome.

It was to be expected that reagents would penetrate to different extents at different stages of an instar. Animals were, however, never used just before or just after a moult, since fixation then distorted them badly, and no consistent differences in iron distribution were found at other stages of the instar (the stages being judged by the developmental stages of embryos in the brood-pouch).

The distribution of loosely-bound iron in the tissues is seen in table 1. All individuals in a given population did not give identical data, but the large majority were consistent. Immature animals, however, were so variable that they were discarded. The blue staining of gut contents was, of course, ignored. The main results deduced from the table are the following.

(1) *Colourless animals*, in well-aerated water. These contained least iron, which was confined to gut-wall and fat-cells, equally in the ferrous and ferric states.

(2) *Animals gaining haemoglobin*, i.e. changing from colourless to red after being put from aerated into oxygen-deficient water. Iron was now found in many organs, particularly in the ferrous state; it was most concentrated in gut-walls, fat-cells, and ovaries. Some was in the prominent sarcoplasm (Binder, 1932) of antennal and mandibular muscles. It looks as if iron were being absorbed through the gut-wall to accumulate in fat-cells and ovaries. These

nay thus be the sites of haemoglobin synthesis. The fat-cells may supply aemoglobin to the blood, and while it is true that the ovaries receive haemo-lobin from the blood (Fox, Hardcastle, and Dresel, 1949), the pigment may e synthesized in them as well. The iron was in the non-yolky part of the vary, in the 4-cell groups.

(3) *Red animals*, living in poorly aerated water. The iron distribution and oncentration was similar to that in group (2), with the small differences seen n table 1.

(4) *Animals losing haemoglobin*, i.e. changing from red to colourless after eing put from poorly into well-aerated water. There was now an intense

TABLE 1

Distribution of loosely-bound iron in Daphnia magna

Staining: +++ intense blue, ++ blue, + pale blue, . no blue.

	(1) *Colourless animals*		(2) *Animals gaining haemoglobin*		(3) *Red animals*		(4) *Animals losing haemoglobin*	
Approximate number studied	200		200		200		400	
Valency of iron	Ferrous	Ferric	Ferrous	Ferric	Ferrous	Ferric	Ferrous	Ferric
Mid-gut walls								
ant. 1/3	.	.	+	.	+	.	.	.
post. 2/3	+	+	++	.	++	.	+	+
Gut caeca walls	.	.	+	++	+	+	+	+++
Fat-cells	+	+	++	+	++	++	.	+++
Shell-glands	.	.	.	.	.	.	.	+++
Ovaries	.	.	++	.	+	.	.	.
Blood-plasma	.	.	+	.	+	.	.	.
Appendage muscles	.	.	+	.	+	.	.	.

No iron was found in fore-gut-wall (oesophagus), hind-gut-wall (rectum), blood-cells, heart-muscle, septa between blood spaces, nervous tissue, lens of eye, integument, or vestigial antennary glands.

Prussian blue colour of ferric iron in the gut caeca, the fat-cells, and the shell-glands, with less iron than previously in the rest of the mid-gut-walls and none in the ovaries. These are striking changes. Less iron in the gut-wall means that less was being absorbed, no doubt because haemoglobin was no longer being synthesized. The absence of iron from the ovaries may well be caused by the cessation of haemoglobin synthesis there. The preponderance of ferric iron in (4), in contrast to that of ferrous iron in (2) and (3), corresponds to the oxygen levels in the water.

The most striking change, however, in (4) is the strong concentration of iron in the shell-glands, where it had previously been absent. The presence of abundant iron in this pair of excretory organs when *Daphnia* is losing haemo-globin, and at no other time, suggests strongly that iron is being excreted after haem has been broken down.

Table 1 (4) also shows a very considerable concentration of iron in the fat

cells. It has already been seen that this tissue may be a site of haemoglobi synthesis, but the present data suggest that it is also a place of haemoglobi destruction. It seems as if iron were liberated here from haem, to be carrie by the blood-stream to the shell-glands for excretion. That the fat-cells a actually a site of haemoglobin removal from the blood in animals becomi pale is confirmed by a discovery of Green (1955*b*). *Daphnia* is occasionall found with red fat-cells and the microspectroscope shows this to be th colour of oxyhaemoglobin. Green found that in a population losing haemo globin, if few eggs are produced, owing to malnutrition, the fat-cells becom red, whereas if eggs are abundant the fat-cells are not red. It is clear tha abundant eggs remove most of the haemoglobin, whereas without enough egg haemoglobin accumulates in the fat-cells until they have time to break down.

Finally, in table 1 (4) we see a high concentration of iron in the walls of th gut caeca. Thus this, too, may be a site of iron excretion, although in (2) iro seemed to be absorbed in this place, to be used in haemoglobin synthesis The same site for absorption and excretion is not improbable, just as the fat cells seem to serve both for synthesis and destruction of haemoglobin.

Table 1 has shown the distribution of loosely-bound iron in *Daphnia*. A regards firmly-bound iron, it has already been said that after its unmasking b acid alcohol, tests are unreliable owing to diffusion of the iron from its origina site. Yet such tests gave two results worth recording. One was that firmly bound iron is usually found in the labral glands, whereas loosely-bound iro was never found there; the significance of this is unknown. The other poin is more interesting. Colourless animals, deprived of algal food for a week showed little or no iron in their tissues. Put for a week into water containin *Chlorella*, but no added iron, they gained some iron. When iron was added t the water they showed a more intense Prussian blue after another week. Thu *Daphnia* absorbs iron, when the element is available, without needing it fo haemoglobin synthesis.

In some mammals stored iron is in the form of ferritin, a compound o ferric iron with a protein (Granick, 1942). This can be crystallized and thu demonstrated (Laufberger, 1937). The tissue, for example horse spleen, i teased on a slide with 10% cadmium sulphate solution, and octahedra crystals form in 3 minutes. It was not found possible to get these crystals fro *Daphnia*, nor could ferritin be extracted and crystallized from *Daphnia* b Granick's method (1942). This is, however, not surprising since ferritin is no demonstrable even in all mammals (Michaelis, 1944).

The Excretion of Iron

The conclusion reached above that iron is excreted through the shell glands by *Daphnia* when losing haemoglobin is supported by three lines o evidence.

1. *Location and state of iron in the shell-glands.* Each shell-gland consist of a closed internal end-sac, the *saccule*, a long winding tube, the *labyrinth*

nd a terminal loop, the *vesicle*, opening to the exterior (Claus, 1875). The hell-glands are bathed by circulating blood. Iron was found both in the ellular walls and in the lumen of the labyrinth, but only in the lumen of he vesicle. No iron was present in the saccule walls or lumen. This points o iron being excreted from the walls into the lumen of the labyrinth, to pass hrough the lumen of the vesicle out of the body. In the walls of the labyrinth vere large and small irregularly shaped blue masses, whereas the lumen ontained a non-granular blue liquid which could be moved along the tube by entle pressure on the specimen.

2. *Rate of appearance and disappearance of iron in the shell-glands.* Experiments were made with *Daphnia* which was first cultured in water poor in

TABLE 2

Time of appearance of iron in the shell-glands of Daphnia magna *losing haemoglobin*

Days in aerated water	*Number fixed*	*Number with iron in shell-glands*
1	10	0
2	30	0
4	15	13
6	30	17

TABLE 3

Time of appearance and disappearance of iron in the shell-glands of Daphnia magna *losing haemoglobin*

Days in aerated water	*Colour of blood*	*Number fixed*	*Number with iron in shell-glands*
3	red	10	7
4	red	20	20
10	pink	10	8
15	pale pink	10	6
16	very pale pink	10	3
18	colourless	13	0
19	colourless	5	0

oxygen to make the animal red and then caused to lose haemoglobin in aerated water. Samples from the cultures losing haemoglobin were fixed at intervals and stained for loosely-bound iron. The results of two experiments are shown in tables 2 and 3. The first of these tables shows that iron did not appear in the excretory organs until 3 or 4 days after transfer of the animals to aerated water. Yet the haemoglobin concentration begins to decrease during the first day (Fox and Phear, 1953, fig. 2). Apparently, therefore, several days are needed for the transfer of iron to the shell-glands in detectable quantity. After 4 days most animals had iron in the shell-glands but after 6 days the proportion was less. Table 3 records a longer experiment, continued until the animals had lost all visible haemoglobin. Here there was iron in the excretory organs of some

animals after 3 days, and of all after 4 days. From then onwards progressively fewer had iron in the shell-glands: it had been excreted. When the animals became colourless there was no longer any iron in the excretory organs; this agrees with table 1.

3. *Autoradiography*. Radioactive iron, ^{59}Fe, as ferric chloride solution, was added to *Daphnia* culture water. The Geiger counter showed that it was taken up by the animals, which presumably incorporated some of it into the haemoglobin, as there was evidence of radioactive iron in extracted blood. Autoradiographs (Bourne, 1952) were made of individuals in the various conditions of gaining, losing, or maintaining steady the haemoglobin. Only in those which were losing haemoglobin was radioactive iron seen in the shell-glands, and then quite clearly.

Haem Iron

In addition to the so-called loosely-bound and firmly-bound iron discussed above, *Daphnia* also contains iron incorporated in haem. Haem compounds can be localized histologically by their property of acting as a peroxidase. A classical reagent for showing a peroxidase in tissues is benzidine, which in the presence of a peroxidase and hydrogen peroxide is oxidized to an unstable blue and finally a brown substance. Reduced cyanol (Lison, 1938) has been found to be superior to benzidine, as the blue is more intense and stable. Dunn's modification (1946) of this method was used in the present work. Dunn states that his method shows the presence of haemoglobin, but I have found that all haem compounds tested give the blue colour with cyanol, that is to say, haemoglobin, haematin, pyridine haemochromogen, and haemin, but not bile pigments or iron salts. A tissue showing as strong a cytochrome spectrum as the heart of the crab *Carcinus maenas* (L.), was found to be positive to cyanol, whereas the claw muscles of the crab, in which cytochrome is not seen with a microspectroscope, were negative. With *Daphnia* the cyanol technique was used on whole specimens and the results obtained for tissues other than blood were confirmed with sections.

Cyanol staining gave the following results with *Daphnia*. Colourless animals showed no reaction, or scarcely any, for all the tissues, whereas red animals gave a positive reaction, not only in blood, eggs, and fat-cells, where haemoglobin is already known, but also in muscles, both of appendages and heart, in the anterior part of the mid-gut-wall including the caeca, and, strange to say, in the lenses of the eye. The nervous tissues gave a less intense reaction. Pink animals gave weaker reactions than red ones. If the cyanol staining here shows haem compounds and not another unknown peroxidase, then it is clear that the haem in the tissues varies in quantity, like the haemoglobin of the blood, inversely as the external oxygen concentration. Since these observations were made, Fox (1955) has found spectroscopically that the muscles of *Daphnia* contain both haemoglobin and cytochrome, which vary in concentration with the blood haemoglobin, and that the same applies to haemoglobin in nerve ganglia. We may thus conclude that the cyanol staining did show the presence

f haem. Fat-cells gave a more intense reaction to cyanol when haemoglobin vas being lost than at other times, and this agrees with the work of Green uoted above. In muscles cyanol showed the haem to be in the fibres, not in he sarcoplasm.

Conclusions

The main conclusion that emerges from this work is that when red *Daphnia agna* loses haemoglobin and so becomes pink, pale, and then colourless, as a esult of moving from oxygen-deficient into aerated water, abundant iron is ound in the shell-glands, whence it is excreted. At the same time abundant ron is evident in the gut caeca, where it may also be excreted, and it appears kewise in the fat-cells. We know already (Green, 1955*b*) that the fat-cells are he place where haemoglobin is removed from the blood, and we now have videncethat in the fat-cells haemoglobin is broken down with the liberation f iron. After some time the iron appears in the shell-glands, and it must be ransported there from the fat-cells by the blood-stream. Muscles and nervous ssue lose haemoglobin at the same time as the blood when red *Daphnia* gets nto aerated water, but we do not know if this haemoglobin is broken down *in itu*, or is first transported to the fat-cells for destruction. The second alterna- ve seems probable since iron was not found to accumulate in muscles.

The excretion of iron by *Daphnia* provides a striking contrast to the events n mammals, where the iron derived from the continuous destruction of aemoglobin is conserved and used for further synthesis of the blood-pigment. t first sight it would seem that *Daphnia* is wasteful of iron, but this is pro- ably not so, for relatively much more haem is destroyed in a short time than n mammals, and an accumulation of such quantities of iron might produce a athological condition of haemochromatosis.

When haemoglobin is increasing in *Daphnia magna* in poorly aerated water, ron is found mainly in the mid-gut-walls, fat-cells, and ovaries. In the gut- alls the iron is no doubt being absorbed into the body, while in the fat-cells nd ovaries it is being used for synthesis of haemoglobin.

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ormal and Induced Degeneration of Abdominal Muscles during Metamorphosis in the Lepidoptera

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With 2 plates (figs. 2 and 3)

Summary

Certain segmental units of the three main longitudinal muscle-bands in the abdomen the larva of *Galleria*, *Platysamia*, *Telea*, *Antheraea*, and *Samia* (*Philosamia*) do not generate during the histolytic phase in the prepupa and early pupa. In the 3rd dominal segment the amount of muscle that persists is variable; in the 4th, 5th, and h segments, invariable. Apart from single pairs of transverse muscles in the 2nd and d segments and those of the gut and heart there are no other muscles in the pupa. stiges of degenerated muscles are often found in the pupa.

The longitudinal muscles which survive the transformation of the pupa into the lult degenerate during the first 2 days of adult life.

Experiments were made on larvae, prepupae, pupae, and adults in attempts to in- ence muscle-degeneration and muscle-persistence. Extirpation of ganglia or sever- ce of nerves in larvae and prepupae of *Galleria* caused the normally persistent uscles to degenerate during pupation. Controls in which larvae were dissected before pation revealed no degeneration of denervated muscles. In saturniids denervation o resulted in degeneration or atrophy but only after a much longer period, a matter several weeks instead of several days. Muscles may be affected by extirpation of nglia or severance of nerves in segments preceding their own segment.

Previous workers have shown that the growth of the new adult muscles is dependent the influence of the central nervous system. This is not so in the case of sheets of e muscle-fibres lying under the epidermis of the adult. They develop in the absence central innervation.

Operations which had no effect on muscle-degeneration in the adult included ex- pation of ganglia in pupa and adult, beheading and bleeding, extirpation of corpora lata plus corpora cardiaca, ligations, extirpation of pupal gonads, and isolation of adult domens. Substitution of blood from diapausing pupae or saline for the adult blood isolated abdomens was effective in slowing the process of muscle-degeneration. This sult shows that the blood composition is of importance in the process of histolysis in e adult.

The previous work on the physiology of insect histolysis is briefly reviewed. The fluence of the nervous system as described in this paper is discussed and related to nilar findings in other arthropods and in vertebrates.

Introduction

THE transformation of the caterpillar into the adult moth involves drastic changes in anatomy; these are particularly striking in the case of the uscular system. In some groups of insects, such as the Coleoptera, many uscles pass through the upheaval of metamorphosis unchanged or are trans- rmed *in situ* into the adult type of muscle (Breed, 1903). In other groups, ich as the higher Diptera (Perez, 1910), all the larval muscles may be

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destroyed completely and the adult muscles arise from myoblasts. In the Lepidoptera most of the larval muscles are destroyed but a few remain, apparently unchanged, throughout metamorphosis and can be seen in the young adult (Hufnagel, 1918). They degenerate in the adult.

There are no terms in existence to describe the various types of muscles according to the time of their breakdown. The following new terminology is therefore proposed. Muscles that degenerate during the life of the insect will be described as *caducous*, in the sense of *destined to die*. Muscles that degenerate in the pupa or imago will be called *pupicaducous* and *imaginicaducous* respectively. Muscles that persist throughout life would be termed *non-caducous*. The terms could be applied to other tissues and other stages, e.g. *prepupicaducous*.

Although there are relatively few records from the Holometabola of muscles passing over from the larva to the adult, a number of cases have been described in which there are striking differences in the rates of degeneration of various muscles. Usually the muscles of the abdomen persist for the longest time. In *Thymalus* (Coleoptera) Breed (1903) describes abdominal muscles which pass unaltered from larva to adult. Judging from his descriptions some of these muscles correspond to imaginicaducous muscles in the Lepidoptera. They are the inner layers of the body-wall musculature in all but the first and last abdominal segments. Murray and Tiegs (1935) report similar findings in *Calandra* (Coleoptera) in which the inner layer of fibres in the longitudinal bands of the abdomen and certain other muscles persist to the adult stage. They later disappear, but Murray and Tiegs do not state how soon the disappearance takes place; they merely record that they are absent from adults several weeks old. In the Diptera there are several accounts, not only of delayed histolysis of abdominal muscles during metamorphosis as in *Drosophila* (Robertson, 1936) and *Calliphora* (Perez, 1910), but of persistence into the adult as in *Culex* (Roubaud, 1932; Hulst, 1906) and *Psychoda* (Schmidt, 1928).

A similar phenomenon is the degeneration of flight-muscles in the adults of various orders. The histolysis of the imaginicaducous flight-muscles of queen ants is well known from the work of Janet (1907), but it also occurs in Isoptera (Feytaud, 1912), Aphidae (Homoptera) (Johnson, 1953), various Coleoptera (Jackson, 1933, 1952), Diptera (Mercier, 1920, 1924, 1928), and probably in Dermaptera (Mercier and Poisson, 1923) and aquatic Hemiptera (Ferrière, 1914; Poisson, 1924). Ewer (1954) reports that certain muscles of the thorax in Acrididae (Orthoptera) degenerate in the adult.

The following account deals with anatomical and experimental studies on the imaginicaducous muscles of several species of Lepidoptera.

Material and Methods

The insects used in this study were *Galleria mellonella* (the wax-moth) and several species of Saturniidae. Larvae and pupae of *Platysamia cecropia* (reared in Connecticut) were kindly supplied by Professor C. M. Williams. Pupae of

Telea polyphemus and *Samia walkeri* (*Philosamia cynthia*) were obtained from dealers in the United States and later these two species were raised in the laboratory in Birmingham. *Antheraea pernyi* was reared in the laboratory in Birmingham.

The surgical techniques used have been developed by Williams (1946, *a* and *b*) and included the use of continuous carbon dioxide anaesthesia and of phenylthiourea (Williams, 1952) to inhibit tyrosinase which causes blackening of the blood on exposure to the air. The precursors of the melanin are toxic. After operations larvae were either kept under anaesthesia for several hours or stored overnight at 5° C. These procedures apparently allow closing and drying of the wound to take place while the animal is immobile, and reduce the mortality from bleeding. Large incisions in silk-moth larvae were sewn with nylon or cotton thread, dried with a stream of dry air, and sealed with melted paraffin wax. Isolated adult abdomens were tied with fine string; the raw tissue was cauterized and then coated with paraffin wax.

Except for a few experiments with *Galleria* the animals were kept at 25° C and 70% relative humidity. The humidity in the cages or jars containing feeding larvae on foliage would be much higher than 70%, but the operated animals in the spinning, prepupal, pupal, and adult stages were kept at 70% R.H.

The Larval–Pupal Transformation

The muscular system of a lepidopterous larva is extremely complex. Lyonet's (1762) classical description of the anatomy of the larva of *Cossus* includes a very detailed account of the musculature. Fortunately for this study only a few of the abdominal muscles are left intact during the formation of the pupa and they are easily located. If a caterpillar is opened along the mid-dorsal line and pinned out flat, and the alimentary canal and fat body are removed, the innermost layers of the muscles of the body-wall are exposed. These are the three pairs of longitudinal bands which stretch the full length of the animal. Each band consist of a series of segmental units attached to the intersegmental groove and to their homologues in preceding and succeeding segments. By cutting the connexions to the body-wall these longitudinal muscles can be removed as continuous strips; this demonstrates that the units in each segment are firmly connected to their fellows. They will be called the dorsal, lateral, and ventral longitudinal muscles. The dorsal bands lie close to the heart and are broad. The lateral bands lie ventral to the line of spiracles. They are also broad. The ventral bands are narrow and lie fairly close to the nerve-cord. In the thorax the positions of the bands alter and overlapping occurs. The lateral bands from the 3rd abdominal segment forwards converge towards the midline and in the thorax they cover the ventral bands.

Dissections of larvae, prepupae, and early pupae show that certain segmental units of the longitudinal bands remain, apparently unchanged, during the process of pupation. The units are those of the 3rd, 4th, 5th, and 6th abdominal segments. Important variations occur in the 3rd segment, both

between species of moth and between individuals of one species. These variations will be described below.

In addition to the longitudinal muscles there are transverse muscles in the 2nd and 3rd abdominal segments of the pupa of *Telea*. Probably they are present in other species as well. They are shown in fig. 1.

The disintegration of the pupicaducous muscles begins in the early pupa; e.g. in *Platysamia* kept at 25° C they have completely broken down about 4 days after pupation. Examination of pupae at successive intervals during the period of histolysis revealed that it does not proceed at a uniform pace through-

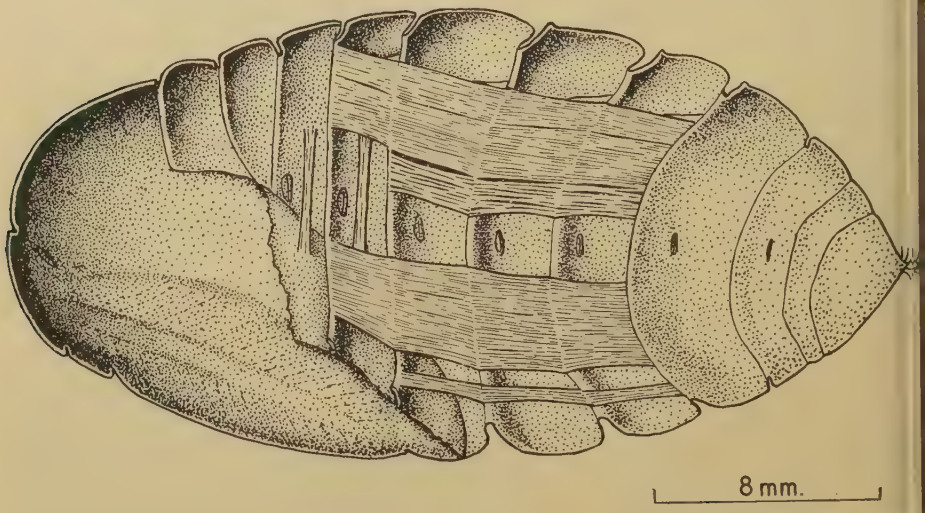

FIG. 1. Diagrammatic representation of right side of male pupa of *Telea* showing the abdominal muscles. In this example there are imaginicaducous muscles in the dorsal and lateral bands of the 3rd abdominal segment.

out the body. Degeneration begins at the anterior end and travels backwards, and probably a corresponding wave from the posterior end travels forwards. The two waves of degeneration never meet in the case of the three longitudinal bands; they stop at the 3rd or 4th abdominal segment and the 6th abdominal segment respectively. The longitudinal muscles of the 4th, 5th, and 6th abdominal segments invariably persist (fig. 1), but in the 3rd segment this is not the case. The ventral band of the 3rd segment never persists in *Telea* or *Platysamia*. The lateral band of the 3rd segment may be present in its entirety or it may have degenerated completely during pupation. In *Telea* it is probably present in less than half of all pupae, similarly in *Platysamia*, but in *Antheraea* it is present in most pupae. Unfortunately, precise statistics are not available because it has not been possible to sacrifice a large number of pupae to provide the necessary figures. The dorsal band of the larva is clearly subdivided longitudinally into three component bands. In the pupa all three of these bands are present in the 4th, 5th, and 6th segments (fig. 1), but in the

d segment of *Telea* and probably of other species also, one, two, or three of e subdivisions may be present or all three may have degenerated.

Frequently there are vestiges of degenerated muscles in the pupa. They are ost commonly found in the 3rd segment in those cases where the lateral or e dorsal bands have degenerated during pupation. Fig. 3, F (opposite p. 222) a photograph of such a vestige from the lateral band of the 3rd segment of *alleria*. The myoplasm disappears but the nuclei may persist in a filmy sheet obably composed of the partially broken-down sarcolemmas and tracheoles. seems that histolysis may be halted before the degeneration of the muscles complete. Occasionally vestiges may be visible in other segments of the dy. They are present in most or perhaps all pupae.

The dorsal longitudinal muscle-band of the pupa of *Telea* consists of two yers of fibres. The fibres of the innermost layer stretch from one intersegental fold to the next and are linked with their fellows of neighbouring segents. There are approximately 45 fibres on the inner surface as viewed in a ssection. The outer fibres are slightly oblique; they diverge slightly from e midline as they run posteriorly. These slightly oblique muscle fibres run e full length of the segment but are not linked with muscles in other segents. Each layer varies between 1 and 2 fibres in thickness. The lateral band approximately 35 fibres in width and 3 in thickness. The ventral band is out 10 fibres in width; the total number of fibres is about 16. The only other uscles in the abdomen of the pupa are those on the wall of the gut, the alary uscles of the heart, and the intrinsic heart-muscles.

The Pupal–Adult Transformation

After diapause has been broken by chilling (Williams, 1946*b*) the pupa of *elea* moults but does not cast the pupal cuticle. The retraction of the epiermis, which is one of the earliest signs of renewed development, can be seen hrough a plastic window placed on the tip of the abdomen or the face Williams, 1946*b*), or it may be seen through the cuticle in the leg or face by vetting the cuticle with 70% alcohol. The retraction of the leg or facial epiermis witnessed through the cuticle is not apparent until approximately 4 hours after the retraction can be clearly seen through a plastic window Schneiderman and Williams, 1954). From the time of the first signs of reraction until emergence of the adult moth takes 3 weeks at 25° C. During this eriod the pupal muscles continue to be functional. The pupa twirls its bdomen when disturbed and in an enclosed space such as its cocoon it can pin round and round.

During the period of adult development many new muscles are formed and become attached to the body-wall, the nerve-cord, the gut, and the genitalia. The term *neoblastic* will be used to describe them. Nüesch (1953) has described the muscles of the thorax in the adult of *Telea*. In the abdomen the imaginicaducous bands are extended in front and behind by the growth of neoblastic muscles. Neoblastic muscles also grow outside the imaginicaducous

muscles, between them and the epidermis. The neoblastic muscles differ fro the imaginicaducous muscles in appearance. They are transparent, where the imaginicaducous muscles are opaque. The opacity is due, not to cloud ness of the myoplasm, but to the extremely abundant tracheation. Numero tracheoles filled with air give the muscles their white appearance and rend them much more conspicuous than the neoblastic adult muscles (fig. 3, E This excessive tracheation is indicative of a higher oxygen requirement by t persistent muscles than by the new muscles (Wigglesworth, 1954), but the is no evidence that it is correlated with their histolysis. The observation Grosch (1952) that the posterior, more heavily tracheated part of the salivar gland of *Microbracon* (*Habrobracon*) degenerates before the anterior part ma be pertinent. Most of the new muscles are composed of thin fibres but tw longitudinal bands which are formed immediately adjacent to the nerve-cor are thick and strong (fig. 2, A and B). They are used to curl the abdomen durin copulation and oviposition. Other neoblastic muscles are the cutaneous. The will be described later.

The imaginicaducous muscles degenerate within about 2 days of emergenc at 25° C leaving the adult with only the neoblastic muscles. The imaginica ducous muscles play an important role in the newly emerged adult. They ar largely responsible for producing the pressure of the blood which causes th wings to unfold and expand. During the process of wing expansion the abdome pumps vigorously, driving blood into the wings. After the degeneration of th imaginicaducous muscles the moth is incapable of such violent contractions o the abdomen. Kuwana (1936) reports a similar degeneration of muscles in th adult of *Bombyx* and Hufnagel (1918) in *Hyponomeuta*.

After degeneration of the imaginicaducous muscles has taken place a single narrow fibre becomes visible in each segment of the dorsal band (fig. 2, B). Closer study revealed the presence of these fibres in most abdominal segment of the pupa as well as in the adult. Each fibre is richly innervated and is probably a stretch proprioceptor. Detailed studies of their structure and function ar being carried out.

Control of Muscle Degeneration during Pupation

The problem posed by the events described above is clear-cut. During the process of pupation most of the larval muscles undergo histolysis and disappear completely or leave only slight vestiges; but by contrast certain segmental units of the three main longitudinal muscles do not break down at this stage. How do these members of a series of units differ from their homologues that do break down during pupation? The muscles that persist may be intrinsically different from the corresponding muscles in other segments or the

Fig. 2 (plate). A, dissection of newly emerged adult female *Telea* showing imaginicaducous longitudinal muscles in 4th, 5th, and 6th abdominal segments. The neoblastic, cutaneous muscles are visible also.

B, dissection of adult male *Telea* about 4 days after emergence. The imaginicaducous muscles have disappeared.

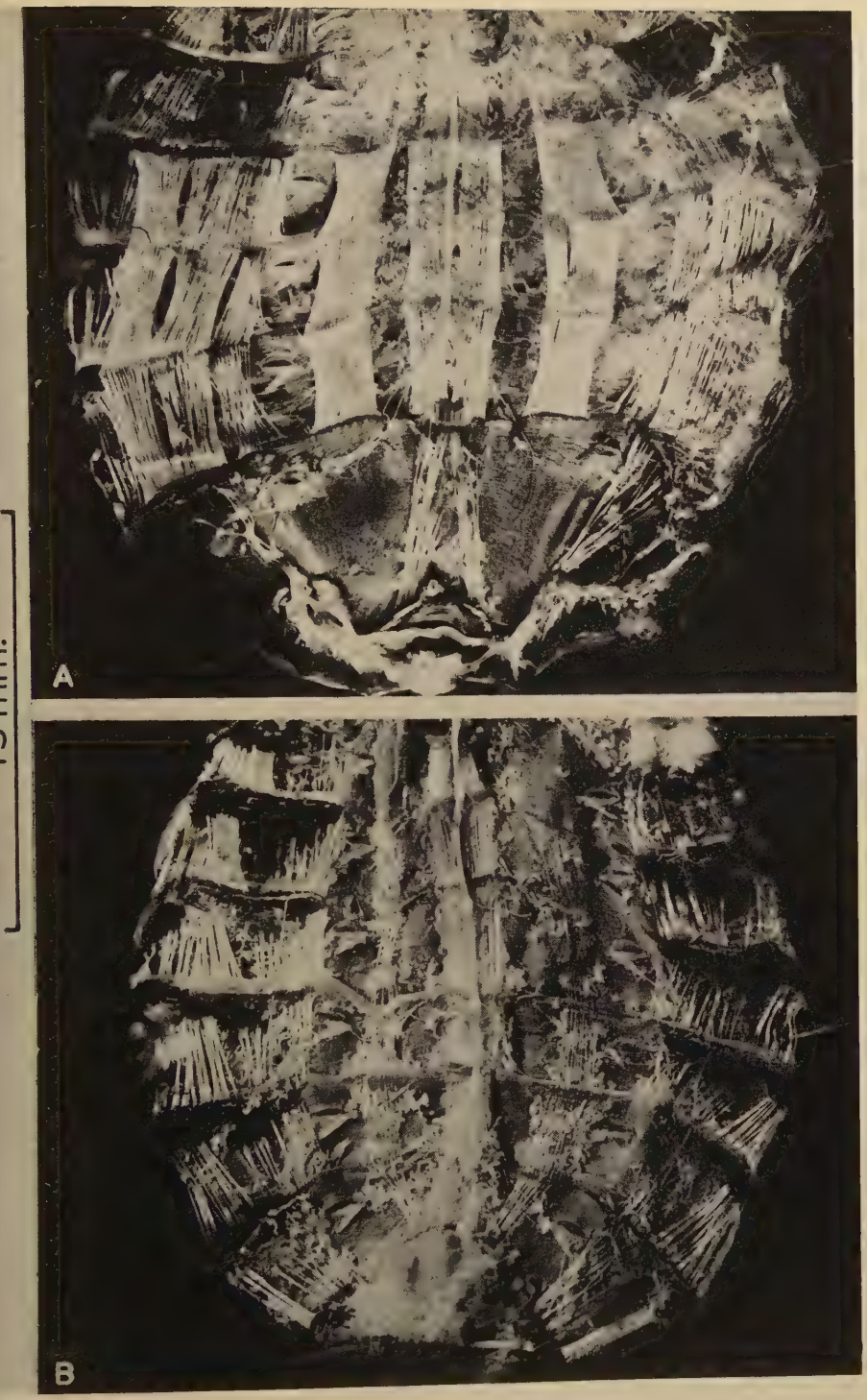

FIG. 2

L. H. FINLAYSON

ontrol of histolysis may be extrinsic and the muscles of all segments identical n properties. The first hypothesis seems to me to be the less likely one. The nuscles of all segments look identical and presumably they arise as members f a single series in the embryo. The most striking evidence against the hypo-nesis of intrinsic differences is the fate of the segmental units of the dorsal and ateral bands in the 3rd abdominal segment. As described above, considerable ariation occurs in this segment in the amount of muscle that may be pre-erved. It seems to me that this is strong evidence for supposing that some actor outside the muscles determines which will persist amid the destruction oing on around them. Undoubtedly the hormone balance of the blood is the hief factor in the initiation of histolysis of larval tissues but there must be nother factor involved in the histolysis of the muscles in question, a factor hat varies in a discrete fashion in the 3rd abdominal segment. The most likely ariable is the innervation. The great importance of the nervous system in the rowth and regeneration of muscles in the vertebrates is well known, and in he insects it was shown by Kopeć (1923) and confirmed by Williams and Schneiderman (1952) and Nüesch (1952) that innervation is essential for de-elopment of the major muscles of the adult moth.

Ganglionectomy and denervation of Galleria mellonella

During the winter months saturniid caterpillars were not available and xperiments were carried out on *Galleria*. Various abdominal ganglia were emoved, or the main nerves were severed close to their origin from various bdominal ganglia, in last-instar feeding larvae, spinning larvae, and pre-upae. After the usual post-operative period at 5° C they were placed at 25° C r 30° C and 70–76% relative humidity and kept under these conditions until hey had pupated, and for variable periods thereafter. Then they were dis-ected. Some larvae were dissected as controls before they pupated. Altogether 0 successful operations were carried out, of which 10 were controls.

The controls were dissected after 4–8 days at 25° C and 7–12 days at 30° C table 1). In all except one the muscles were complete and active in segments , 4, 5, and 6. The segmental numbers of the ganglia which were removed are hown in table 1. The exception was a larva which fed for 11 days at 25° after aving ganglion A5 removed, and which showed slight atrophy of the lateral muscles in segments 5 and 6.

The 40 experimental animals consisted of 18 feeding larvae, 12 cocoon spinning larvae, and 10 prepupae at the head-flexed stage (Bounhiol, 1938). They were dissected after periods ranging from 2 to 5 days after pupation. Usually 1 or 2 ganglia were removed but in a few cases 3 or 4 were removed. Results were similar regardless of the stage of the insect when the operation was carried out. The muscles in the segment deprived of its ganglion or in the side of the segment which had been denervated became very thin and then in-visible in the living animal within 2–4 days from pupation, and after fixation with Bouin only showed up as vestiges similar to those frequently found in segments where the larval muscles have degenerated in the normal course of

events (fig. 3, F, G, and H). The normal muscles of the pupa (really the developing adult by this time) are thick and yellowish and easily stimulated to contract. The denervated muscles at best are very thin and transparent and the ability to contract is lost soon after pupation. The innervation of the muscles was not studied directly but the results of these experiments shed some light on the course of the segmental nerves. In many cases (see table 2) not only the

TABLE 1

Galleria *controls*

Temperature	*Ganglia removed*	*Number of days from operation until dissection*
25° C	A5	4
	A5 and A6	6
	A5 and A6	6
	A6	7
	A5	8
	A6	8
	A2	8
	A5	11 (slight atrophy of laterals in 5th and 6th segments)
30° C	A4 and A5	7
	A4 and A5	12

muscles in the segment deprived of a ganglion but those in the following segment degenerated in the pupal stage.

The results shown in table 2 were selected from the latest series of experiments made on *Galleria*. The operations were carried out with special care to eliminate the possibility that the degeneration of muscles posterior to the excised ganglia was due to accidental damage to the nervous system.

The results of the experiments on *Galleria* show clearly that denervation of the imaginicaducous muscles results in their degeneration in a few days after the onset of metamorphosis but not during the larval stage. Denervation of

FIG. 3 (plate). A, part of abdominal body-wall of adult, female *Platysamia* showing the neoblastic, cutaneous muscles. The spiracles of the 3rd to 7th segments are visible. (The 7th segment bears the last spiracle of the adult abdomen.)

B, section of neoblastic (left) and imaginicaducous muscle (right) from adult *Telea*. The imaginicaducous muscle is degenerating (haematoxylin and eosin).

C, section of normal muscle from left, lateral band of 5th segment of pupa of *Platysamia* (table 4, no. 11) (haematoxylin and eosin).

D, same animal, right lateral band of 5th segment. Denervated muscle showing degeneration (haematoxylin and eosin).

E, living muscles from newly emerged adult *Telea*. Neoblastic muscle below, imaginicaducous muscle above. Note the greater tracheation of the latter.

F, whole mount of lateral muscle band from 4th, 5th, and 6th abdominal segments of pupa of *Galleria*. Ganglion A5 was removed in the prepupal stage (table 2, no. 3). Extreme degeneration in 6th segment, less severe in 5th segment but still very pronounced.

G, same animal, whole mount of vestigial muscles from lateral band of 3rd segment after normal degeneration (haematoxylin).

H, same animal, whole mount of vestigial muscles from lateral band of 6th segment of opposite side showing degeneration induced by denervation (haematoxylin).

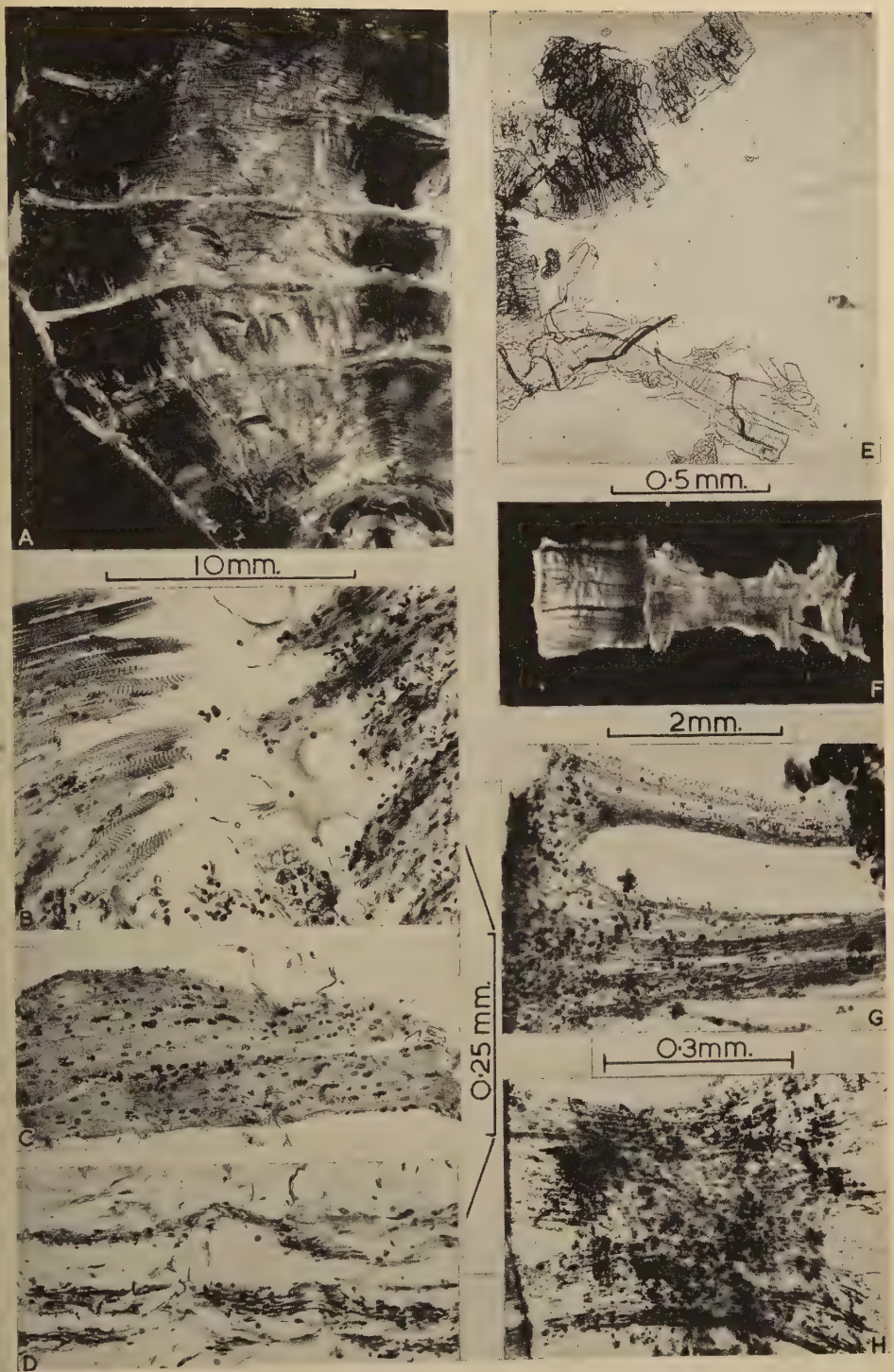

FIG. 3

L. H. FINLAYSON

both larvae and prepupae leads to premature degeneration in the pupa. At 25° C the induced degeneration of the imaginicaducous muscles is not obvious until 2 days after pupation. The breakdown of the rest of the larval musculature has already taken place during these same 2 days.

TABLE 2. *Ganglionectomy of larvae and prepupae of* Galleria

Stage	Number of days from: operation to pupation	Number of days from: pupation to dissection	Ganglia removed	Muscles affected
Prepupa	2	3	A4	A4, A5
Prepupa	1	4	A4	A4, A5
Prepupa	1	4	A5	A5, A6
Prepupa	1	4	A6	A6
Larva	8	3	A5	A5, A6
Larva	7	2	A4	A4, A5
Larva	5	2	A3	A4
Larva	5	2	A5, A6	A5, A6
Larva	16	3	A2	None

Ganglionectomy and denervation of saturniid larvae and prepupae

Experiments identical to those on *Galleria* were made on larvae and prepupae of the diapausing silk-moths *Antheraea pernyi* and *Platysamia cecropia*. The results are shown in tables 3 and 4. Although denervation and ganglionectomy eventually lead to degeneration of the imaginicaducous muscles in the

TABLE 3. *Ganglionectomy and nerve section in final instar larvae of* Antheraea

Number	Stage	Number of days from: operation to pupation	Number of days from: pupation to dissection	Total	Ganglia involved	Muscles affected
1	spinning	7	6	13	A4, A5	None
2	spinning	8	10	18	*A5, *A6	None
3	feeding	8	16	24	A4, A5	None
4	feeding	18	9	27	A4, A5	None
5	feeding	19	9	28	A4*, *A5	None
6	feeding	17	11	28	*A4*, *A5*	A4, A5
7	feeding	21	9	30	A4	A5, A6
8	spinning	5	25	30	A3, A4	A3, A4, A5
9	spinning	6	25	31	*A3*, *A4*	A3
10	feeding	7	29	36	*A4*, *A5*	A4, A5
11	feeding	6	38	44	*A5, A6*	A5, A6
12	spinning	8	37	45	A4, A5	A4, A5, A6
13	spinning	9	64	73	*A3*	A3
14	feeding	10	67	77	A4, A5	A4, A5, A6
15	spinning	8	72	80	*A3, *A4	A3, A4
16	spinning	8	82	90	*A4*, *A5*	A4, A5, A6
17	feeding	7	109	116	A5, A6	A5, A6
18	spinning	7	112	119	*A4, A6*	None

* Indicates nerves severed close to ganglion, including the branch from the median nerve of the preceding ganglion. The position of the * signifies left or right side of ganglion.

Where the ganglion numbers alone are given the ganglia were removed entirely.

pupa, the time-lag is very great and of a completely different order from the short period of a few days which elapses between pupation and the first obvious signs of muscle degeneration in *Galleria*. In *Antheraea* (table 3) there was no

TABLE 4

Ganglionectomy and nerve section in final instar larvae and prepupae of Platysamia

Number	*Stage*	*Number of days from:*		*Total*	*Ganglia involved*	*Muscles affected*
		operation to pupation	*pupation to dissection*			
1	spinning	4	1	5	A3, A4, A5, A6	None
2	prepupa	3	3	6	A4, A5, A6	None
3	spinning	11	3	14	A3, A4, A5	None
4	spinning	9	5	14	*A4, *A5, *A6	None
5	spinning	5	10	15	A4*, A5*	None
6	prepupa	3	15	18	A1–A8 (incl.)	None
7	prepupa	4	17	21	T3–A8 (incl.)	None? (muscle fibres thin)
8	spinning			51	A1–A5 (incl.)	None?
9	spinning			66	T3–A6 (incl.)	A3 vestige, A4, A5, A6 early degeneration
10	prepupa	5	119	124	*A3, *A4, *A5, *A6	Left side: A3, A4, A5, A6
11	spinning	11	118	129	A3*, A4*, A5*, A6*	Right side: A3, A4, A5, A6
12	spinning	14	119	133	A3, A4 (or A4, A5)	A4, A5, A6 early degeneration
13	prepupa	10	130	140	*A3*, *A4*, *A5*	None. Nerve regeneration?
14	spinning	9	177	186	*A3, *A4, *A5*, A6*	Left side: A3, A4, A5, A6 Right side: part A6

* See table 3.

sign of degeneration before 28 days from the operation (11 days from pupation) and even then there were at least a few fibres in the bands which could still contract, although they were much thinner than normal. Even after 116 days (no. 17) the muscles were not completely broken down. They were soft, thin, heavily tracheated, and non-functional, but still discrete and obvious fibres. After normal degeneration, which takes only a few days, very flimsy vestigial fibres may be left, but usually the remnants, if any, are not recognizable as separate fibres. They form a fenestrated, flimsy sheet probably composed of remnants of sarcolemmas held together by remnants of the tracheal system.

In *Platysamia* the story is similar (table 4; fig. 3, C and D). At 21 days and 1 days after operating there was doubt whether degeneration had begun. robably the muscle fibres in these two cases (no. 7 and no. 8) were thinner han normal but in no. 8 there was a curious complication in that many of the ormally pupicaducous muscles had failed to degenerate. After 186 days no. 14) the denervated muscles had degenerated considerably though scarcely o the degree that pupicaducous muscles degenerate within a few days during upation.

Multiple innervation of persistent muscles

The results of the denervation experiments indicate that the imaginica- lucous muscles are innervated by nerves from more than one ganglion. The anglia anterior to the segment containing a muscle-band may contribute to

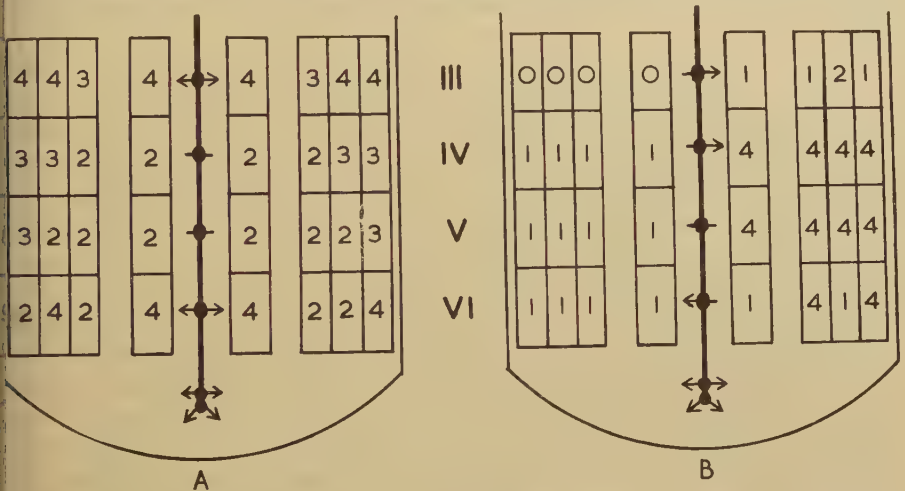

FIG. 4. Diagrams representing lateral and dorsal muscle-bands of the 3rd to 6th abdominal segments of A, *Antheraea* (table 3, no. 16), and B, *Platysamia* (table 4, no. 14). The arrows indicate nerves left intact, elsewhere all nerves were severed close to the ganglion. The numbers 0–4 indicate amounts of muscle visible. 0 = complete degeneration, 4 = normal muscles.

its innervation, but there is no indication that ganglia in segments posterior to that containing the muscle supply any innervation. The experiments on *Galleria*, *Antheraea*, and *Platysamia* all support this observation. One specimen of each of the two saturniid species is particularly interesting. In *Antheraea* no. 16 (table 3) all the main nerves from the vicinity of ganglia A4 and A5 were severed. The lateral bifurcations of the median nerve from ganglion A3 and ganglion A4 were severed but the median of A5 was left intact. The result is shown diagrammatically in fig. 4, A. Both lateral and dorsal muscles in the 4th and 5th segments degenerated as expected. In the 3rd segment the laterals were complete and the dorsals were represented by the two upper subdivisions. In normal animals this is a common pattern for the 3rd segment. In

the 6th segment the laterals were complete but the dorsals were represented by only one of the three sub-divisions on each side. On the left it was the middle one which was normal and on the right it was the most dorsal. In *Platysamia* no. 14 a comparable result was obtained (fig. 4, B). In *Antheraea* the muscles of the 6th segment were affected by severing nerves from ganglion A5. In *Platysamia* some muscles in the 6th segment remained intact despite the absence of innervation from ganglia A5 and A6. They were maintained presumably by innervation from ganglion A4. Of course there is the possibility of regeneration of nerves from one of the ganglia or a new growth of branches from ganglion A4 reaching the dorsal muscles in segment A6. Regardless of whether the effect of differential degeneration in the dorsal band shown by these two animals is influenced by the primary innervation or by regenerated nerves, the fact remains that by interfering with the innervation it is possible to produce a result which mimics the normal state of affairs in the 3rd segment.

The Pupal–Adult Stage of Saturniids

The muscles which have persisted throughout the larval–pupal metamorphosis and the pupal–adult metamorphosis degenerate in the young adult. Again there is differential behaviour of muscles: the imaginicaducous degenerate and the neoblastic adult muscles remain intact.

Normal degeneration of the persistent muscles in the adult

Emerging adult silk-moths were kept under observation and the time of emergence was noted as precisely as possible. The adults were kept at 25° C and 70% R.H. for various periods and then dissected. The state of the persistent muscles was noted. The observations were made on 18 *Telea*, 8 *Platysamia*, and 8 *Samia*. The results show that degeneration of the muscles begins during the first 24 hours of adult life and is usually complete by 48 hours (fig. 2, B; fig. 3, B). It is not easy to decide when degeneration has just begun but when it is under way the difference between the persistent and new adult muscles is clear. The imaginicaducous muscles become flabby and incapable of contraction. After fixation the difference is even more obvious.

Experiments designed to influence muscle degeneration in the adult

A number of experiments were carried out on saturniids in attempts to prevent breakdown of the muscles or alter the rate at which they degenerate. These experiments will be described briefly.

Removal of ganglia from pupae and newly emerged adults. Various ganglia were removed from nine chilled pupae of *Telea* and three *Platysamia* which were then allowed to develop into adults and dissected after various periods of adult life at 25° C and 70% R.H. In three *Telea* the entire abdominal nerve cord was removed. The imaginicaducous muscles remained throughout adult development and then degenerated in the usual way in the young adult. Although the main abdominal neoblastic muscles were absent from adults pro-

luced from pupae deprived of all abdominal ganglia, the cutaneous muscles vere present.

Removal of head from adult. The newly emerged adult expands its wings by orcing blood into them. Most of the pressure required is obtained by the ction of the imaginicaducous muscles of the abdomen. It seemed significant hat the muscles begin to degenerate after they have performed this function. t was thought that the muscles might not degenerate if this behaviour pattern vas not carried out. Removal of sufficient blood from a newly emerged adult revents wing expansion.

Three *Platysamia* which had been developing at 25° C for 20–21 days and vere due to emerge at any moment were removed from the pupal cuticle and eheaded. A large proportion of the blood was removed at the same time for nother purpose. The wings failed to expand. After 4 days at 25° C the per- istent muscles had degenerated completely. Thus the removal of the head nd prevention of wing expansion by bleeding did not prevent degeneration f the muscles.

Ligation between thorax and abdomen, or severance of abdomen. This experi- nent was a counter-check on the preceding one and it was planned to demon- trate, in addition, whether there was any factor produced in the thorax which nfluenced the degeneration of the persistent muscles. One adult *Platysamia* vas ligated and three adults had their abdomens removed and sealed with araffin wax within 1 hour of emergence. After approximately 4 days at 25° C nd 70% R.H. in the case of the ligated animal and 3 days in the others, the maginicaducous muscles had degenerated completely.

Removal of corpora allata and corpora cardiaca. Although the experiments n which the head was removed or the abdomen isolated rule out the possi- ility of the corpora allata having an immediate influence on muscle degenera- ion, a few removals of the corpora allata were performed on chilled pupae of *Telea* to make sure that they were not acting at any time during the develop- nent of the adult. The corpora cardiaca were removed with the corpora allata. Adult development was allowed to proceed. One animal was dissected before he moulting fluid had been resorbed and the imaginicaducous muscles were still intact. The other three animals were dissected 26, 32–56, and 48–96 hours fter emergence. In all three adults the muscles had degenerated completely. Therefore the normal behaviour of the muscles in question can take place in he absence of the corpora allata and cardiaca from the pupal stage onward.

Removal of gonads. The testes were removed from a male pupa and the varies from a female pupa of *Platysamia*. In both cases the imaginicaducous nuscles degenerated in the normal way.

Replacement of adult blood by pupal blood or saline. Abdomens removed from ewly emerged adults were bled as completely as possible and in the case of emales most of the eggs were removed also. The abdomens were then filled p with blood from diapausing pupae of *Samia* and firmly ligated. This peration is difficult and the results cannot be accepted as conclusive. In the ew preparations which survived, the degeneration of the imaginicaducous

muscles appeared to have been delayed. Even after 106 hours degeneration was not complete. The results are shown in table 5. In most of the survivors there was some leakage of blood from the isolated abdomen and it is possible that the presence of the imaginicaducous muscles after their normal limit of 48 hours was simply a consequence of the shortage of blood for removing the products of histolysis. A number of abdomens survived but were so desiccated

TABLE 5

Adult blood replaced by diapausing pupal blood or saline

Species	*Sex*	*Number of hours from:* emergence to operation	*Number of hours from:* operation to dissection	*State of muscles*
		DIAPAUSE BLOOD		
P. cecropia	male	1½	46½	Intact, thin, contractile
S. walkeri	female	2	46	Degenerating
P. cecropia	male	1½	63½	Degeneration almost complete
S. walkeri	male	3	66	Intact, thin
P. cecropia	male	1	95	Degenerating
T. polyphemus	female	not emerged	106	Degenerating
		SALINE*		
S. walkeri	female	1	43	Relaxed fibres
T. polyphemus	female	1	54	Degenerating
T. polyphemus	male	1	70	Degeneration almost complete
S. walkeri	female	1–12	72	Relaxed but still contractile
S. walkeri	female	1	72	Relaxed, dorsals contractile?
S. walkeri	female	1	76	Slight degeneration of laterals; dorsals normal
S. walkeri	female	1–16	88	Relaxed, non-contractile
T. polyphemus	male	1	91	Degeneration complete
S. walkeri	male	1–16	96	Degeneration complete

* Formula for saline: NaCl, 7·5 g; KCl, 0·35 g; $CaCl_2$, 0·21 g; H_2O, 1 litre. (Ephrussi and Beadle, 1936.)

that they were not recorded as successful preparations. In some of them the persistent muscles were present but obviously dead and merely left *in situ*.

Similar experiments in which the adult blood was replaced by insect Ringer gave better results (table 5). The persistent muscles took longer to degenerate than normally.

THE CUTANEOUS NEOBLASTIC MUSCLES

In the adult silk-moth there are fine muscle-fibres forming a band along each side of the abdomen from the 1st to the 6th segment. In the 7th-10th segments, which form a single unit undivided internally by intersegmental folds,

ıe fibres form a continuous sheet right across the ventral region of the ɔdominal body-wall (figs. 2, A and 3, A). Histologically the fibres appear to ɛ typical striated muscle. The function of these muscles is unknown and no revious reference to them can be traced.

It is known that the major neoblastic muscles of the adult (fig. 2, B) depend n the presence of innervation from the central nervous system for their de-ɛlopment (Kopeć, 1923; Williams and Schneiderman, 1952). If the central ervous system is removed from a pupa the resulting adult lacks these muscles. he cutaneous muscles of the silk-moths, however, develop normally in the bsence of central innervation.

Discussion

Histolysis in insects has been treated in great detail from the descriptive, istological aspect (reviewed by Oertel, 1930; Snodgrass, 1954), and much ttention has been focused on the role of the phagocytes. The present in-estigation is concerned with the gross aspects of muscle histolysis and the ɔntroversy about phagocytes will not be revived at present. The little we now about the physiology of histolysis is due to the work of Bodenstein. He nowed (Bodenstein, 1943*a*) that the larval salivary glands of *Drosophila* ndergo histolysis under the influence of the ring-gland but only when they ave reached a certain stage in development. They must be 'competent' to eact to the stimulus of ring-gland of the mature larva just as the imaginal isks must be 'competent' before they will respond by growing (Bodenstein, 943*b*). The critical factors in both involution and growth are the hormone alance of the blood and the sensitivity of the tissue itself. The relationship etween hormonal content of the blood and histolysis of the larval salivary lands in *Drosophila* seems logical and straightforward, but in the case of the uscles which I have studied no obvious correlation can be seen between the istological events and the hormonal situation. Muscles degenerate during upation but also during adult life. In the former case the corpora allata have topped secreting and the prothoracic glands are active. In the young adult he corpora allata are active and the prothoracic glands have long since dis-ppeared (Williams, 1952). This does not mean that there is no prothoracic land hormone remaining in the blood, but if present it is likely to be in very ow concentration. Another difficulty in correlating muscle histolysis with ormones is the fact that in the larva parts of an apparently homologous series f intersegmental bands undergo histolysis while other parts remain un-ffected, both in the same environment.

Bodenstein (1953, *a* and *b*) also studied the degeneration of the prothoracic lands in *Periplaneta* and found that the corpora allata and the corpora ardiaca are involved. In the presence of the corpora allata alone or both orpora allata and corpora cardiaca the prothoracic glands degenerate after 2–14 days of adult life. In the absence of the corpora allata and in the presence f the corpora cardiaca the prothoracic glands behave abnormally and do not

degenerate in the adult. The corpora allata are present and active throughout larval life when the prothoracic glands are present. Again we can see no straightforward relationship between the activity of the corpora allata and the persistence or involution of the prothoracic glands. As Bodenstein postulated the effects of the various glands must be achieved by shifts in the balance between their secretions. Similarly, the process of muscle-degeneration in so far as it is controlled by hormones must also be related to hormone balance rather than to the influence of one or other hormone.

The 'trophic' action of the nervous system on muscle has been studied chiefly in the vertebrates (Tower, 1939; Bowden, 1954), but the work of Needham (1945, 1946, 1950, 1953) on *Asellus* has established its importance in regeneration and for the maintenance of muscle in the Crustacea. Needham (1946) found that denervation of limb-muscles resulted in atrophy which he regarded as disuse-atrophy. In the Amphibia, Singer (1952) considers that atrophy in the adult produced by denervation may be a similar process to regression in a denervated regenerate and not simply a result of disuse. Sustet (1933) demonstrated the need for innervation in the regeneration of muscle in the leg of *Sphodromantis* (Orthoptera). Roeder and Weiant (1949) saw no signs of degeneration of denervated muscle in *Periplaneta* after a period of 14 days, but the results obtained with *Platysamia* and *Antheraea* suggest that a longer period might be necessary to produce an obvious effect. The work of Kopeć (1917, 1923) is more directly applicable to the present study. Kopeć removed ganglia from the thorax of the pupa of *Lymantria* and found that the resultant adult had no muscles in the thorax. This phenomenon was rediscovered by Williams and Schneiderman (1952) and studied in further detail by Nüesch (1952) in giant saturniid moths. Williams and Schneiderman stated that removal of the abdominal ganglia from pupae results in the adults having no abdominal muscles. Presumably the imaginicaducous muscles had degenerated before the dissections were carried out or they had atrophied during the period of denervation and were therefore almost invisible in the living condition. Kopeć also removed the 5th abdominal ganglion from young 3rd instar larvae which he succeeded in rearing for 3–5 weeks before examining the muscles in microscopical sections. He found that the muscles of the 5th segment deprived of its ganglion were normal and he concluded that lack of innervation had no effect even though it had lasted for about three-quarters of the larval life-span. Kopeć does not consider the possibility of re-innervation of the muscles in the 5th segment from other ganglia to make up the complement of nerves needed to maintain them in the normal state. The innervation supplied by the 4th abdominal ganglion would in any case be unaffected by the operation. In view of the results obtained with saturniids, in which prolonged denervation of the pupa produced muscle atrophy and the indication that prolonged denervation of *Galleria* larvae may have the same effect, the claims of Kopeć require stronger evidence. Technically it is difficult to rear a young larva successfully after extensive denervation. Even if the larva survives it is difficult to establish that no re-innervation has taken place. There seems little

ubt that the influence of the nervous system during the larval stage is less pressive than during pupation. This lack of influence by the nervous system reminiscent of the apparent absence of influence of the nervous system in e vertebrate embryo (Singer, 1952).

The development of the cutaneous neoblastic muscles of the adult in the sence of the central nervous system appears contradictory to the hypothesis at innervation is essential for neoblastic muscle development. However, e cutaneous myoblasts, although deprived of central innervation, are not ecessarily deprived of all nervous connexions. As in many insects (Wiggles-orth, 1953) peripheral neurones are present under the epidermis. They can be evealed in *Telea* by methylene blue staining. Presumably they are sensory, but is does not rule out the possibility that they induce development of the utaneous muscles. It has been established for the vertebrates that sensory as ell as motor innervation can promote regeneration (Singer, 1952). Needham 953) has suggested that cutaneous neurones in Crustacea may influence egeneration and quotes in support of this suggestion the work of Suster 933), who found that the epidermal derivatives of the regenerating leg of *phodromantis* were not dependent on central innervation. Suster's results xtended the findings of Kopeć (1917, 1923) for *Lymantria*.

The results of the ganglionectomy and denervation experiments with *Galleria* and saturniids show that the nervous system must be taken into ccount as a possible factor in the control of muscle histolysis. In *Galleria* the esults are more convincing than in the saturniids because of the rapid rate of egeneration of the denervated muscles during pupation and the similarity in eneral appearance between the vestiges of muscles which have degenerated ormally and those which have been deprived of their innervation. In the aturniids the denervated muscles take a long time after pupation to show any igns of degeneration and even then the appearance of the fibres is more haracteristic of atrophy than histolysis and may be simply a result of disuse. hese differences between the experimental animals may not be fundamental ut a reflection of the differences in metabolism between the non-diapausing *Galleria* and the diapausing saturniids. Even in *Galleria* the denervated im-ginicaducous muscles remain normal in appearance for longer than the pupi-aducous. The two waves of histolysis have normally just reached the maginicaducous muscles when the histolysis phase comes to an end. In the aturniids the onset of diapause may halt the histolytic processes more abruptly han in the non-diapausing wax-moth. The presence of substantial remnants f muscles in the pupae of both types of lepidopteron confirms that when istolysis is stopped in the early pupa, any muscles which have not already isappeared are left in varying stages of degeneration. An observation by owden (1954) that muscular atrophy following denervation is more rapid in hildren than in adults may be relevant to this discussion. The more rapid de-eneration is found in the actively growing individuals.

The histolysis of muscles in the young adult cannot easily be explained on n innervation hypothesis. To do so we should have to postulate a selective

withdrawal of neural influence from the imaginicaducous muscles and not from the neoblastic muscles. This is a stage further than the supposition that during pupation the preserving influence of the nervous system is withdrawn from whole segments. It is possible to visualize the innervation of the imaginicaducous muscles by persistent neurones in the central nervous system whose influence fades in the adult, but there is no foundation for such speculation.

If the nervous system should prove to play a part in normal histolysis the problem would be moved one stage back and become the problem of the intrinsic differences between parts of the nervous system, rather than a problem of differential reactions by various parts of the muscular system to the stimulus of the hormone balance at metamorphosis. The importance of the hormones is recognized and the experiments on substitution of the blood of adults by diapause blood or saline indicate that hormones may be playing a part in histolysis in the adult or at least that the condition of the blood is important.

The natural variability of the muscles in the 3rd abdominal segment of the pupa supports the innervation hypothesis, and the results from several denervated animals in which parts of muscle-bands were left intact also lend support. There may be a dosage effect similar to that found in vertebrate regeneration (Singer, 1954), in which the quantity of nerve tissue in the regenerate is critical. In the 3rd segment it would appear that only the ganglion of that segment can influence the muscles and variation therefore occurs in the amount of muscle preserved because the nerve-dosage is near threshold. The variation is commonest in the dorsal band which is farthest from the ganglion. The lateral muscle-band is either 'hit' or 'missed' completely by the preserving influence. In the 4th, 5th, and 6th segments there is neural reinforcement from a preceding segment or segments. In the absence of ganglia in the 4th and 5th segment the muscle of the 6th segment may show a partial degeneration similar to that normally seen in the 3rd segment, presumably for the same reason.

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The Blood Vascular System of Nephtys (Annelida, Polychaeta)

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Summary

The four longitudinal vessels of the circulatory system of *Nephtys californiensis* are rsal, sub-intestinal, and neural, the latter being paired. There is a complete longi-dinal circulation; the dorsal vessel communicates with the sub-intestinal by way of e proboscidial circulation and with the neural by way of the circum-oral vessels. In ch middle and posterior segment segmental vessels from each of the longitudinal nks carry blood to and from the parapodia and body-wall. The segmental cir-lation is completed by a circum-intestinal vessel connecting the dorsal and sub-testinal vessels in each segment and an intersegmental branch connecting the dorsal d sub-intestinal segmental vessels. A trans-septal branch of the neural segmental ssel communicates with the sub-intestinal segmental vessel. This arrangement is odified in anterior segments which house the muscular, eversible pharynx, and no ood-vessels cross the coelom except by running through the body-wall. On anatomi-l grounds and by comparison with other polychaetes it seems likely that segmental subordinate to longitudinal circulation. There are no endothelial capillaries such as ave been described in some other polychaetes; instead there are numerous blind-nding vessels the walls of which are composed of the same three layers as other vessels nd which are probably contractile. The dorsal vessel, where it is in contact with the entral surface of the supra-oesophageal ganglion, forms a plexus in close association ith a modified part of the brain capsule and a special axonal tract within the ganglion. t is thought that by way of this 'cerebro-vascular complex', hormones produced in ne neurosecretory cells of the brain pass into the blood-stream.

Introduction

THERE has never been a detailed study of the blood vascular system of any member of the Nephtyidae, a family of polychaetes which is of some mportance in that it is probably one of the least specialized in the class. The irculatory system of *Nephtys caeca* Fabricius has been described by Ehlers 1864–8) and by Schack (1886) and that of *Nephtys hombergi* Audouin and Milne Edwards by Milne Edwards himself (1837), by Jaquet (1886), and by le St.-Joseph (1894). When these accounts are compared, they are found to be ull of mutually incompatible observations, and most of them are cursory and ll incomplete, if not inaccurate, in many respects. So little concensus of pinion is there that it is impossible to tell from these accounts whether or not here are any differences between the vascular systems of the two species. However, most of the examinations appear to have been so superficial that nly the grossest differences would have been detected. So far as the main eatures of the circulatory system are concerned, it seems that the two species re essentially the same. A redescription of the vascular system of *Nephtys* is verdue, particularly since the concept of functional morphology was not amiliar to the nineteenth-century anatomists.

The circulatory system of *Nephtys* does not afford any anatomical

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surprises: it follows the general pattern found in other errant polychaetes. There are four main longitudinal vessels: these are the dorsal, sub-intestinal and paired neural vessels. Except in the specialized anterior segments, the four vessels communicate with each other directly; in the anterior part of the worm the dorsal vessel bifurcates to form the circum-oral vessels, which in turn become the neural vessels, while a pair of vessels from the dorsal vessel communicates with the sub-intestinal vessel by way of the proboscidial circulation. Thus there is generally a complete segmental and a complete longitudinal circulation. In the anterior 30 or so segments the segmental circulation is modified. This specialization is related to the presence of the highly muscular eversible pharynx; the anterior segments are nonseptate and no lateral blood-vessels cross the coelom as they do in more posterior, unmodified segments. A further specialization of the anterior vascular system, though not in this case related to the presence of the pharynx, is the elaboration of a 'cerebro-vascular complex' in which a short length of the dorsal blood-vessel forms an intimate connexion with the epithelium on the ventral surface of the supra-oesophageal ganglion. Evidence that by way of this complex, hormones produced in the neurosecretory cells of the brain pass into the blood-stream, will be presented in a later paper. Here, the anatomy of the various parts of the vascular system will be described and the structure of the cerebro-vascular complex will be discussed only in so far as it relates to the arrangement of the blood-vessels.

Materials and Methods

It is possible to follow the course of all the major vessels and of most of the smaller branches by careful dissection under a binocular dissecting microscope. Doubtful connexions between vessels and the course of smaller ones have been confirmed by the examination of serial sections. Since reliable dissections can be made only of freshly killed animals, this study is based for the most part on an examination of *Nephtys californiensis* Hartman, which is readily available between tide-marks in central California. At maturity it reaches a length of some 20–25 cm and is therefore of a convenient size for dissection. It has not been found necessary to use the benzidine reaction (Prenant 1921) as an aid to tracing the finer vessels as Faulkner (1930), Ewer (1941), Hanson (1950), and Bobin (1951) have done. In fact, in many of the specimens the smaller vessels are already coloured with a dark pigment which aids considerably the task of tracing their ramifications. For microscopic examination the worms were fixed in Zenker-acetic, Zenker-formaldehyde, Heidenhain's 'Susa', or Bouin's fluid and the sections stained with Mallory triple stain or a combination of Gabe's (1953) paraldehyde-fuchsin and the trichrome counter-stain of Halmi (1952), which has been developed for the study of neurosecretory cells, but which also gives excellent differentiation of other tissues. Methylene blue, used as a vital stain in the study of the histology of the blood-vessels, was prepared by making a 0·5% aqueous solution which was then filtered and diluted with 25 times its volume of sea-water for use.

The Anatomy of the Vascular System

The whole worm, when fully grown, includes some 120–150 segments. The roboscidial apparatus, consisting of a thin-walled buccal region, a stout, uscular pharynx, and the retractor and protractor muscles, occupies the nterior 35 of them. The first 10 segments are somewhat smaller than the ucceeding ones; so the inverted proboscis occupies one-fifth to one-sixth of ne total length of the worm. For convenience, this modified anterior part of ne worm which houses the extrovert will be referred to as the proboscidial egion.

The dorsal vessel for most of its course lies embedded beneath the muscle oats of the intestine. It is about 0·1 mm in diameter in a fully grown worm nd does not taper appreciably except at the extreme posterior end. As it pproaches the proboscidial region it is thrown into tight folds (segments XL–XXXV) and then, in segment XXXV, it dilates to form a bulb lying at the unction between the intestine and the pharynx. In the course of the next 15 egments it crosses from the gut to the dorsal body-wall and completes its nterior course suspended between the two halves of the dorsal longitudinal nuscle by a fine connective tissue mesentery. In its passage from intestine o dorsal body-wall, the dorsal vessel is thrown into a generous loop which lies reely in the coelom, attached to the intestine at one end and to the mesentery t the other, but unsupported in between. Because of its disposition, the orsal vessel is not visible from the exterior, as is that of *Nereis*, for example, xcept for a few segments anterior to segment XX, where the dorsal longi-udinal muscles do not meet completely in the mid-line and the blood-vessel an be seen indistinctly between them by transparency. In segment II, two ranches of much the same diameter as the dorsal vessel arise from it and un, unattached, back through the coelom to the papillae at the anterior end f the pharynx (figs. 1 and 2). When the proboscis is everted, these vessels un out through the mouth between the thin buccal sheath of the proboscis nd the muscular pharynx, as the dorso-lateral proboscidial vessels. In seg-nent I, at the posterior margin of the supra-oesophageal ganglion, the dorsal essel bifurcates, though its two branches do not immediately separate. nstead, they run side by side, with frequent anastomoses between them, and orm part of the cerebro-vascular complex (fig. 1). This is described in greater etail below. At the anterior margin of the ganglion the two branches diverge nd for a short distance follow the circum-oesophageal connectives, but in the rst segment they leave them to supply the body-wall of segments I and II. he vessels return to follow the connectives in segment II and converge on the ub-oesophageal ganglion, which lies in segment V. These two circum-oral lood-vessels do not meet ventrally, but pursue separate courses, one on each ide of the ventral nerve cord, as the neural blood-vessels.

The sub-intestinal vessel is a little narrower than the dorsal vessel and is lso embedded beneath the muscle coats of the intestine for most of its course. n the proboscidial region this, like the dorsal vessel, is detached and it runs

freely along the ventral surface of the pharynx to the terminal papillae as th ventral proboscidial vessel.

In the anterior part of the worm the dorsal and sub-intestinal vessels com municate with each other by way of the proboscidial circulatory system (se fig. 2). They gave rise to the paired dorso-lateral and ventral proboscidia vessels respectively and when the proboscis is everted, they lie unattache

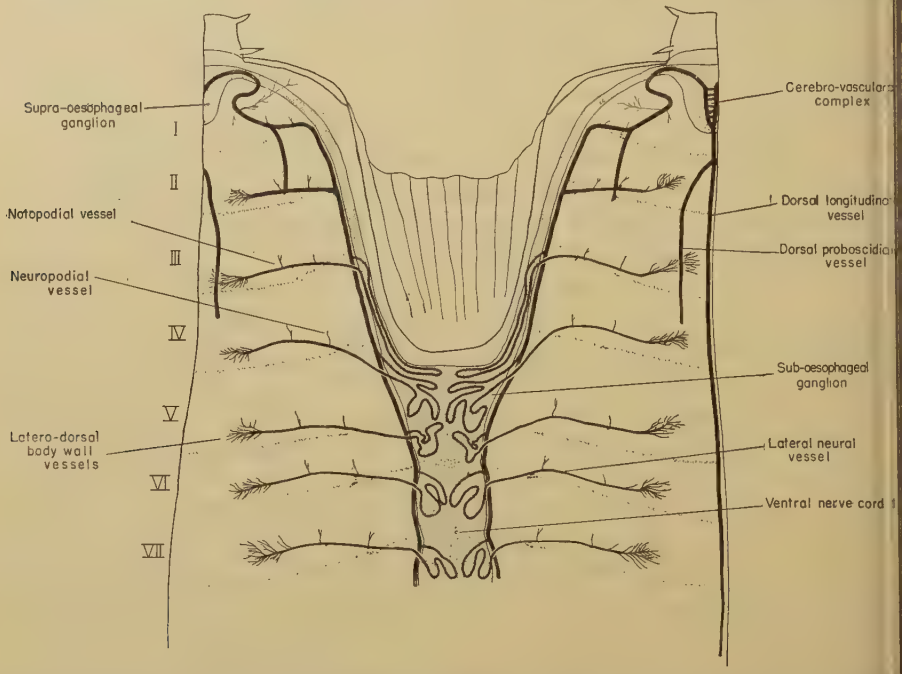


FIG. 1. Dissection of the anterior segments of *Nephtys californiensis* to show the blood vascula system. The worm has been opened by a mid-dorsal incision, the pharynx, and with it th sub-intestinal vessel, has been removed, and the two halves of the body-wall reflected laterally

between the buccal sheath and the muscular pharynx. At the distal end of th proboscis the ventral vessel divides and each branch runs round to the sid of the extrovert. There is a fine anastomosis between the two dorso-latera vessels before they also diverge to run to the sides of the proboscis. Jaque (1886) disputed the presence of this anastomosis, which had previously bee reported by Milne Edwards (1837) in *Nephtys hombergi*. It is rather difficul to see because it frequently drains of blood and becomes invisible when tension is applied to the other vessels during dissection. Possibly this anasto mosis is a regular feature of the blood vascular system of *Nephtys* whic Jaquet overlooked. At the sides of the proboscis the vessels disappear beneat the bases of the papilla muscles to form a complicated ring with loops runnin into each of the eleven terminal papillae on each side of the proboscis. No where does the system break up into capillaries or a fine plexus such as Nicol (1954) described surrounding the muscular extrovert of *Nereis limbata* an

ereis virens. Furthermore, there is not a simple ring vessel surrounding the trovert, connecting the dorso-lateral and ventral proboscial vessels, as chack (1886) described in *Nephtys caeca*, and the proboscidial circulation is mewhat more complicated than, though essentially similar to, that described d figured by Ehlers (1864–8) in the same species.

The vascular system of an unmodified segment, that is one posterior to the roboscidial region of the worm, is illustrated in fig. 3. In each segment aired vessels leave the dorsal, sub-intestinal, and ipsilateral neural vessels supply the body-wall and parapodia. The segmental vessels leave the dorsal ngitudinal blood-vessel in about the middle of the segment and run postero-terally to the septum. In doing so they half-encircle the gut, giving off an

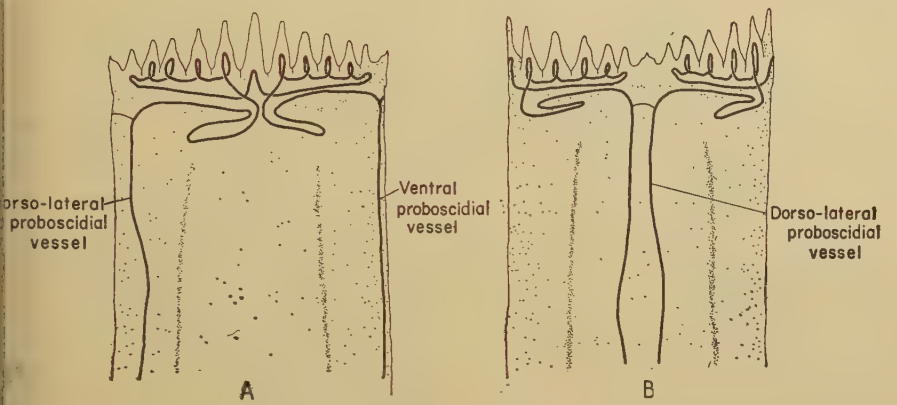


FIG. 2. Proboscidial circulation of *Nephtys californiensis*. A, lateral view; B, dorsal view.

testinal vessel which runs into the sub-intestinal longitudinal vessel, and ass under the lateral edges of the dorsal longitudinal muscles. A fine branch f the dorsal segmental vessel passes dorsally along the outer surface of the ngitudinal muscle, between it and the dorsal parapodial muscles which flank laterally, and ends where the dorsal muscle meets the dorsum of the seg-ent. Along its course, this dorsal vessel arborizes and produces a number of ne, blind-ending diverticula. These are the only type of 'capillary' found in *Nephtys*. Conventional endothelial capillaries have been found in a number f polychaetes (e.g. Nicoll (1954) describes them in *Nereis*, and Hanson (1949) views their occurrence in other polychaetes), but they appear to be missing rom *Nephtys*. These blood-vessels do not penetrate between the longitudinal nd circular muscles on the dorsum of the worm, nor do any blood-vessels enetrate into any of the large muscle masses. At the insertion of the para-odium into the body-wall, the dorsal segmental vessel bifurcates, the main ranch running through the dorsal part of the parapodium to the branchia nd the inter-ramal area; the finer, intersegmental branch runs ventrally, llowing the insertion of the parapodium into the body-wall just in front of he anterior face of the septum. It gives off a great many blind capillaries hich lie in the posterior wall of the parapodium and in the intersegmental

part of the body-wall. The intersegmental vessel finally joins the sub-intestina segmental vessel.

The sub-intestinal segmental vessels, like those of the dorsal vessel, aris mid-segmentally and after partly encircling the gut, run posteriorly an laterally to the septum. They are quite long and loop down into the coelom presumably to allow for changes in the shape of the segment during locomotio of the worm. These vessels pass to the ventral, posterior, lateral corners of th segment and there communicate with the intersegmental branch of the dorsa vessel. They give off a small nephridial branch and also communicate with th trans-septal branch of the neural segmental vessel. The main branch run into the neuropodium where it arborizes.

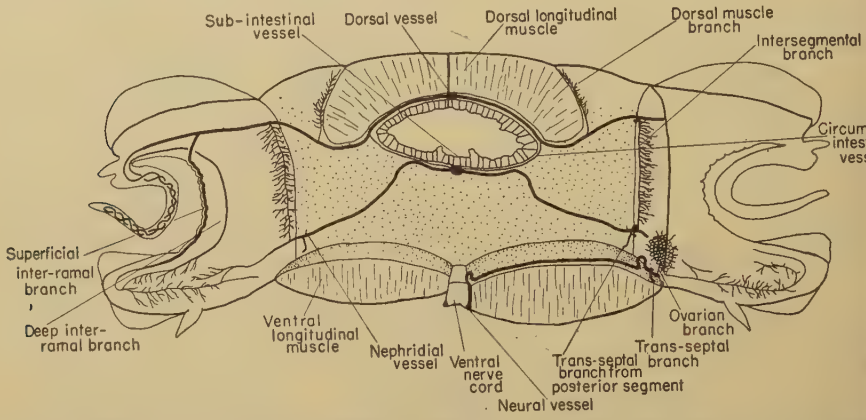
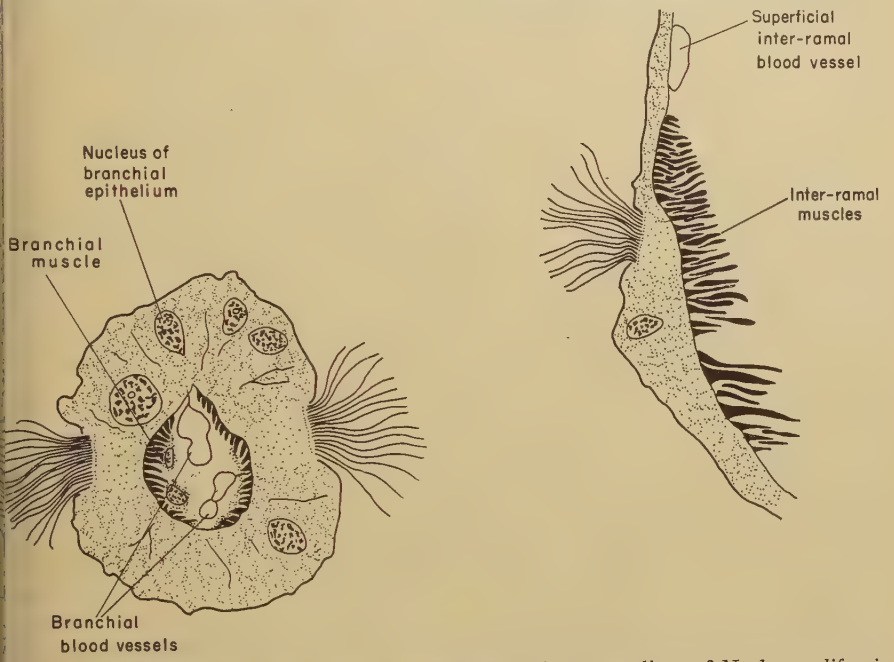


FIG. 3. Schematic view of the circulatory system of a segment from the middle region o *Nephtys californiensis*. The anterior septum has been removed, the posterior one is stippled The anterior half of the segment and parapodium has been removed from the left-hand side

The neural segmental vessels arise in the anterior part of the segment They run up the sides of the nerve cord, are coiled into a small loop lying on top of it, and then run between the ventral longitudinal muscles and th diagonal muscles lying on top of them directly to the base of the neuropodium at its insertion into the body-wall in the anterior part of the segment. Afte coiling into a second loop, the neural segmental vessel disappears between th parapodial muscles. At this point, at the lateral edge of the ventral longitudina muscle, the blood-vessel bifurcates. One branch runs anteriorly through th septum into the segment in front, and communicates with the sub-intestina segmental vessel of that segment. The other branch runs posteriorly a shor distance, gives off a small gonadial vessel, and runs into the neuropodium.

While a significant proportion of gaseous exchange must take place acros the body-wall of the worm, the parapodia are the most important respirator surfaces. The thin-walled branchiae and the inter-ramal areas are heavily ciliate (fig. 4) and the entire parapodium is highly vascularized. There are, as we hav seen, three main blood-vessels entering or leaving the parapodium, one from each of the segmental vessels. The notopodial branch of the dorsal segmenta

essel divides to form the branchial and superficial inter-ramal vessels. A umber of fine vessels from both branches supply the anterior and posterior alls of the parapodium, particularly in the dorsal half, but some extend into e neuropodium. Schack (1886) figures a small vessel in the dorsal cirrus of *ephtys caeca*, but I have not been able to see one in *Nephtys californiensis*. he branchial vessel becomes very narrow and coils within the branchia; it is tached to the branchial epithelium, but apparently sufficiently loosely for the oils to slide fairly freely over each other. The return vessel from the branchia



IG. 4. Frontal section through the inter-ramal region of a parapodium of *Nephtys californiensis* which passes through the branchia. Camera lucida drawing.

uns ventrally to the neuropodium as the deep inter-ramal branch. Both the eep and the superficial vessels give off numerous blind capillaries in the europodium and eventually they communicate with the two ventral vessels, ose from the sub-intestinal and neural segmental vessels. Jaquet (1886) escribed a very fine capillary bed lying in the neuropodial post-acicular imella of *Nephtys hombergi*. He said that it was not easy to see and was requently invisible. After careful examination, I have not been able to find ny such plexus in *Nephtys californiensis*. The parapodia in the middle part f the worm are all highly vascularized; those at the extreme anterior and osterior ends are less so. The vascularization is for the most part in the form f numerous blind capillaries which, in the middle segments, occupy all the pace not taken up with muscles. Those capillaries in the posterior wall of the arapodium and in the intersegmental body-wall are drawn from the inter-egmental blood-vessel, those in the neuropodium from the sub-intestinal

and neural segmental vessels and also from the two inter-ramal vessels. Ther are relatively few blood-vessels in the notopodium.

In every segment, other than those of the proboscidial region, two fin vessels from the dorsal blood-vessel encircle the intestine and communicat with the sub-intestinal vessel. These intestinal vessels are serpentine, pre sumably to permit dilation of the gut, but they do not break up into a plexus There is no vascular supply to the pharynx except for the proboscidial cir culatory system described previously.

The arrangement of the segmental blood-vessels is modified in the anterio

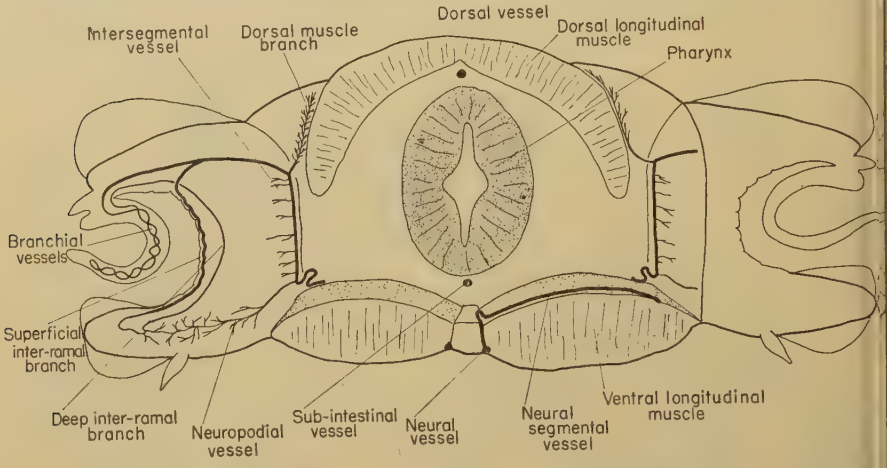


FIG. 5. Schematic view of the circulatory system of a segment from the anterior region o *Nephtys californiensis*. The anterior half of the segment and parapodium has been remove from the left-hand side.

35 segments which comprise the proboscidial region of the worm (figs. and 5). In these segments there are neither dorsal nor sub-intestinal segmenta vessels, but the modifications appear even posterior to this. There are n sub-intestinal segmental vessels anterior to segment L, and between segment XL and XXXV the dorsal segmental vessels are considerably elongated, s much so that those of segment XXXV are twice as long as those of segmer XLV. The intersegmental branch of the dorsal segmental vessel in a typica segment runs into the sub-intestinal segmental vessel, but when the latter i missing (as in segments L–XXXV) the intersegmental branch communicate with the trans-septal branch of the neural segmental vessel. This does n involve the development of any new connexions, since the trans-septal vess communicates with the sub-intestinal vessel and by way of it with th intersegmental vessel, in any case. In the proboscidial segments, where then is neither dorsal nor sub-intestinal segmental vessel, the intersegmental vess assumes a new importance. All the peripheral parts of the vascular system i these segments are the same as in more posterior, typical segments and the onl way in which blood reaches the dorsal muscle vessel and the parapodium by way of the neural segmental and the intersegmental vessels. This becom

large vessel in these segments, of the same diameter as the neural segmental essel, unlike the slender intersegmental in more posterior segments. The sult is that in segments of the proboscidial region, the neural segmental essel crosses the anterior part of the segment between the central longitudinal nd diagonal muscles and passes into the segment in front (there is no septum, o it cannot be described as 'trans-septal' as in posterior segments.) There it oils into a loop before sending one branch into the neuropodium. The main ranch runs dorsally, in the same position as the intersegmental branch of osterior segments, to the dorsal part of the parapodium, where it sends one ranch into the notopodium and another to the dorsal longitudinal muscle. he blood-supply to each parapodium is therefore from the neural segmental essel of the segment behind and there are only two vessels entering the arapodium, one dorsally and one ventrally.

The first five segments are further modified (fig. 1). As in other anterior egments, blood-vessels originating in segments V, IV, III, and II supply he intersegmental area, the parapodia, and the latero-dorsal body-wall of egments IV, III, II, and I respectively. They all take their origin from the ircum-oral vessels, which are, of course, continuations of the neural longi-udinal vessels. The neural segmental vessels originating in segments V and V first run back along the circum-oral vessels to coil extensively over the ub-oesophageal ganglion before running back along themselves to their espective segments. The segmental vessels arising in III and II run directly o segments II and I and are connected by an intersegmental loop of con-iderable diameter at the level of the notopodia. Since the first and second egments are abranchiate, their parapodial circulation is correspondingly educed and modified. Two very fine vessels leave the circum-oral vessels in egment I to supply the lateral lips.

The Adaptations of the Anterior Vascular System

The modifications of the anterior 40–50 segments can be attributed to the presence of the large muscular pharynx and to the fact that it is eversible. There is a considerable relative movement between it and the body-wall through which it passes as the proboscis is everted and, in consequence, there are no dorsal or sub-intestinal segmental vessels in the proboscidial region. In addition, those vessels which are attached to the pharynx, the dorsal vessel at its posterior end and the dorso-lateral and ventral proboscidial vessels at its anterior end, all lie freely in the coelom and are long enough to permit the complete eversion of the pharynx. The anterior part of the intestine must also be stretched as the proboscis is everted and in this region there are no sub-intestinal segmental vessels and the dorsal segmental vessels become pro-gressively longer and longer to allow for the displacement of the intestine relative to the body-wall. The problems posed by the existence of a large, muscular, eversible pharynx are not all solved by the segmental blood-vessels running in the body-wall instead of across the coelom, however. In *Nephtys*

the first 8 or 10 segments are commonly smaller in diameter than the pharynx which has to pass through them as it is everted; when retracted it lies posterior to them. The first 5 segments are specially modified to permit the passage of the proboscis. The ventral floor of these segments is replaced by a muscular gular membrane which is folded and normally tucked within the lateral lips formed by the edges of the lateral walls of these segments. The buccal part of the gut, which forms the thin-walled sheath of the extrovert, is attached to the anterior end of the gular membrane and to the lateral lips. When the proboscis is everted the lateral lips are thrust aside and the gular membrane is tightly stretched. The most extreme distortion of the anterior 5 segments is therefore restricted to the gular membrane, and the sub-oesophageal ganglion lies at its posterior margin, in segment V. The nerves and blood-vessels run in the lateral walls of segments I–IV, which are displaced but not immoderately stretched when the pharynx passes between them.

The segments immediately posterior to V have no such elastic gusset, and the body-wall with its blood-vessels is correspondingly distended by the passage of the pharynx. The neural segmental vessels are coiled into a loop on top of the ventral nerve cord and also at the base of the lateral body-wall, proximal to the origin of the neuropodial vessel. The latter is certainly an adaptation to permit the distension of the body-wall. The blood-vessels are fairly strong and will resist longitudinal tension, but they cannot be stretched, at least not macroscopically. If the body-wall is cut on each side, immediately above the parapodia, and the dorsum removed, these loops are clearly visible, but if the body-wall is stretched in a dissection, the loops disappear. The loops over the ventral nerve cord are more doubtfully concerned with permitting the stretching of the floor of the segment. The body-wall cannot be stretched in such a way as to extend these loops, as the lateral ones can be extended; the diagonal muscles tear away before this stage is reached. Indeed, in one specimen of *Nephtys californiensis* an abnormal anastomosis had been formed between the two loops on each side of the nerve cord in one anterior segment, which would totally have defeated the purpose of these loops did they permit extension of the ventral body-wall. It is more likely that they serve to provide a greater area of contact between blood-vessel and nerve-cord to which they are closely apposed, though not attached, and so serve a respiratory function alone. This idea is strengthened by the elaboration and convolution of these loops in the region of the sub-oesophageal ganglion, which is a good deal thicker than succeeding ganglia.

The Cerebro-Vascular Complex

It is not the purpose of the present paper to describe the neurosecretory system of the supra-oesophageal ganglion, except in so far as it is related to the blood vascular system. The 'cerebro-vascular complex' is a term coined by Bobin and Durchon (1952) for the tract of nerve-fibres, the modified brain capsule, and the blood-plexus at the base of the supra-oesophageal ganglion

hich they described in *Perinereis cultrifera*. Essentially the same relationships re to be found in *Nephtys*, though with some slight differences (fig. 6).

Unlike the supra-oesophageal ganglion of the Nereidae, that of *Nephtys* is pidermal and is in contact with the cuticle on the dorsal surface of the pro-tomium for much of its length. Ventrally the ganglion is bounded by a con-ective tissue sheath which is continuous with the basement membrane of he epidermal cells. It has the same staining properties as this and corresponds o the 'capsule' which completely invests the nereid brain. It is only 2 μ in hickness, except on the ventral surface, particularly in the mid-line, where it hickens considerably. The diagonal prostomial muscles are attached to this hickened part of the capsule in the anterior part of the ganglion.

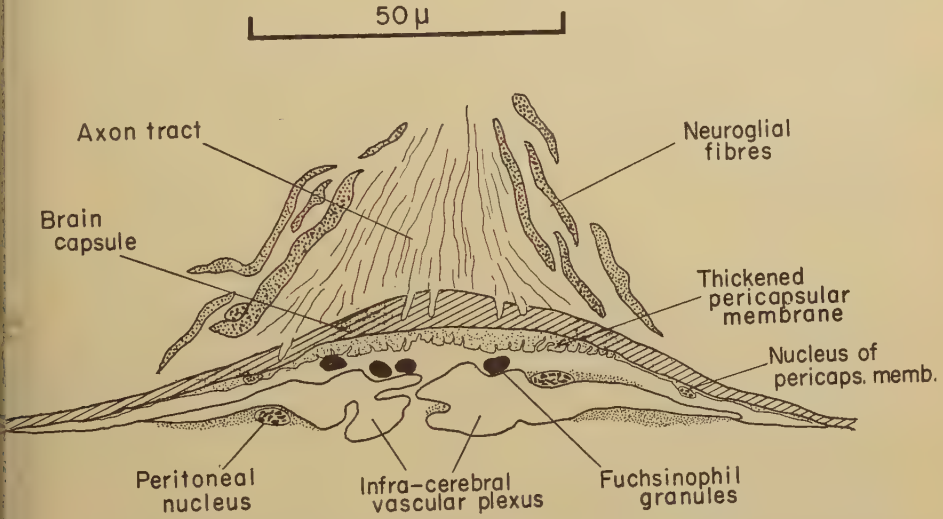


Fig. 6. Transverse section through the cerebro-vascular system of *Nephtys californiensis*. Camera lucida drawing.

Closely applied to the outer surface of the connective tissue sheath is an extremely thin cellular layer, corresponding to the 'pericapsular sheath' of Bobin and Durchon. It can be most easily detected by the small, infrequent nuclei flattened against the connective tissue sheath, but under optimum optical conditions this cellular layer can be seen completely to invest the brain and its sheath. The mid-ventral surface of the ganglion is flattened or even concave and in this depression the pericapsular membrane is thickened to as much as 15 μ. Immediately ventral to the thickened part of the membrane, but separated from it by a gap, lie the paired dorsal vessels which are flattened and between which there are frequent anastomoses. These vessels are supported laterally by fine extensions of the ganglion connective tissue sheath.

Within the supra-oesophageal ganglion a cone-shaped tract of fibres emerges from the neuropile and runs directly to the ventral part of the ganglion and

penetrates into, but not through, the thickened part of the connective tissue sheath immediately above the thickened part of the pericapsular membrane. Bobin and Durchon (1952) have presented evidence that in the cerebro-vascular complex of *Perinereis cultrifera*, neurosecretory granules pass along the fibres in the cone-shaped tract and migrate across the thickened peri-capsular membrane into the blood-vessels. Evidence that the same situation prevails in the cerebro-vascular complex of *Nephtys* will be presented in detail elsewhere. Here it is sufficient to state that fuchsinophil material has been seen in the base of the cone-shaped tract, in the space between the thickened pericapsular membrane and the blood-plexus and around the blood-vessels themselves.

It is thus at least possible that this elaboration of the vascular system on the ventral surface of the brain is an adaptation to provide a large surface-area of blood-vessel for the absorption of hormones released from the supra-oesophageal ganglion. It is unlikely that it represents a respiratory adaptation, because the connective tissue sheath is thickest in the neighbourhood of the blood-vessels, whereas, as we shall show below, in other parts of the nervous system, where blood-vessels are closely applied to the ganglia, the sheath investing them is very thin opposite the blood-vessels.

Circulation and Respiration

The chief contractile vessel, as in all polychaetes, is the dorsal longitudinal vessel. Peristaltic contractile waves normally pass along it from behind for-wards as far forward as the bulb which lies at the junction between the intestine and the pharynx. This bulb has been described as a 'heart' by de St.-Joseph (1894), presumably in the sense that it is the chief propulsive part of the vascular system. In fact, neither it nor the dorsal vessel anterior to it show any but the slightest contractions and it seems likely that it acts as a sort of expansion chamber to equalize the flow into the anterior vascular system. All the blood-vessels, even the fine, blind-ended capillaries, appear to be con-tractile, though none of them show strong contractions at all comparable to those of the dorsal vessel.

The walls of polychaete and oligochaete blood-vessels are commonly com-posed of three layers (Hanson, 1949): 1, an endothelium which may occasion-ally be in the form of a continuous layer, but is often reticulate and possibly sometimes composed of isolated cells; 2, a collagenous connective tissue, or skeletal layer; 3, an outer peritoneal layer differentiated into a muscle coat, or, more often, with contractile fibres in the tails of stellate cells; no contractile fibres have been detected in the peritoneal epithelium on some of the smaller vessels of certain annelids. Retzius (1891) described stellate muscle-cells on the finer vessels of *Nephtys* and by vital methylene blue staining I have been able to repeat his observations. Stellate cells occur on the walls of segmental vessels and the blind capillaries of *Nephtys californiensis*; the dorsal longi-tudinal vessel has a complete muscular coat. Nowhere in the circulatory

ystem are there endothelial capillaries such as are found in *Nereis* (Hanson, 949; Nicoll, 1954). This is strong presumptive evidence that all vessels in the irculatory system are contractile, and while I have not been able to see con-actions of the blind-ending capillaries, I have watched the irregular, inter-ittent contractions of the branchial vessels in parapodia removed from the ody.

Nicoll (1954), in his analysis of the segmental circulation of *Nereis virens* nd *Nereis limbata*, has demonstrated that flow is from the sub-intestinal essel, through the capillary beds of the parapodium, and then medially to the orsal vessel. The return flow from the dorsal vessel to sub-intestinal vessel s by way of an intestinal capillary plexus and a by-pass vessel which short-ircuits the lower half of the plexus.

At first sight it appears from the anatomy of the vascular system of *Nephtys* hat essentially the same segmental circulation may occur in it as in *Nereis*. lowever, the system in *Nephtys* is complicated by two factors. The circum-ntestinal vessels and the intersegmental branch of the dorsal segmental vessel nay be held to be analogues of the intestinal plexus and the by-pass of the ntestinal plexus, which in *Nereis* return blood from the dorsal to the sub-ntestinal vessel. These are narrow and insignificant vessels in *Nephtys* and the uantity of blood flowing through them cannot be great, so that segmental irculation, while not interrupted, must at least be impeded. The second actor which must complicate the segmental circulation is that while in *Nereis* here is no direct connexion between the dorsal and sub-intestinal vessels in he anterior part of the worm, in *Nephtys* there is a pair of blood-vessels of onsiderable size connecting the two by way of the proboscidial circulation. Even in *Nereis*, where there is what one would call, on morphological grounds, complete and direct segmental circulation and relatively imperfect longi-udinal circulation, the segmental circulation is subordinate to the longitudinal irculation. According to Nicoll, if both dorsal and sub-intestinal vessels are igated a few segments apart, the intervening segments are quickly drained of lood. In *Nephtys* where the segmental circulation appears to be relatively ncomplete and the longitudinal circulation extremely well developed, seg-mental circulation must be even more dependent upon longitudinal.

In anterior segments, where dorsal and sub-intestinal vessels are lacking, it s difficult to explain segmental circulation at all. The sub-intestinal segmental essel is to some extent dispensable, since its function is duplicated by the ieural segmental vessel and in fact it is missing from about 15 segments XXXV–L). However, in all the proboscidial segments, the neural vessels lone exist and presumably most of the blood runs to the notopodium and hen back into the same vessel from the neuropodium. Unless there is a eriodic reversal of flow in the neural segmental vessel, it is difficult to imagine ow parapodial blood of these segments can be restored to general circulation, or there are no valves in the blood-vessels; but this reversal has never been bserved. Evidently the circulatory system of these segments is more than heoretically inefficient. The vascularization of the anterior parapodia is much

reduced and the branchiae are often small and frequently lost altogether. In *Nephtys californiensis* all but the first 2 segments carry branchiae, but in *Nephtys punctata* Hartman branchiae are missing from the first 10 segments.

It is surprising that in *Nephtys* there should be such a poor blood-supply to the massive dorsal and ventral longitudinal muscles. In *Nereis*, both sets of muscles have their blood-supply (Nicoll, 1954), and in serpulids and sabellids there are blood-vessels penetrating the dorsal longitudinal muscles (Hanson 1950). Presumably, in *Nephtys* there is a sufficient area of blood-vessels exposed to coelomic fluid for it to act as an important oxygen transport system. Direct gaseous exchange across the dorsal and ventral body-walls, of which these muscles form part, is no doubt also of great importance.

The only structures in the body with a well-developed vascular supply are the gonad and the nervous system. Capillaries penetrate the ovary and project from it in all directions. Thus, not only is the ovary well supplied with blood-vessels within, but the coelomic fluid immediately surrounding it is also probably kept well oxygenated. The gonadial vascular system keeps pace with the development of the ovary so that when it is fully developed and fills practically the whole of the ventral part of the coelom, it has a considerable blood-supply drawn not only from the gonadial vessel, but augmented by capillaries of the intersegmental branch of the dorsal segmental vessel and from the neuropodial vessels. The vascular supply to the ventral nerve-cord appears to be largely incidental and it has no capillary system. In the middle and posterior segments the neural segmental vessels run up the sides of the nerve-cord from the neural longitudinal vessels and where they are in contact with the nerve-cord they are flattened closely against it. The membrane investing the cord immediately under the vessels is exceptionally thin. In the anterior segments the loops formed by the neural segmental vessels above the nerve-cord become elaborate and do not stick up into the coelom, but are flattened and coiled on top of the ganglia. It is in the most anterior segments that the loops become most elaborate and the sub-oesophageal ganglion is invested dorsally, not only with the loops of the segmental vessel of segment V, but also with those of segment IV, which double back from their origin on the circum-oral blood-vessels before running to their appropriate segment. These loops do not appear to have a structural function, but seem instead to be a respiratory device.

This work was carried out while I was an exchange lecturer from the University of Glasgow. It is a pleasure to record the kindness and hospitality I have experienced in the Department of Zoology at Berkeley. I am indebted to the National Science Foundation for a grant in aid and to Mr. James Runner for technical assistance.

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The Distribution of Mast-Cells in the Digestive Tract of Laboratory Animals: Its Bearings on the Problem of the Location of Histamine in Tissues

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With two plates (figs. 1 and 2)

Summary

The mast-cell distribution in the digestive tract of the rat, dog, cat, and guinea-pig as studied. In the rat mast-cells were present in the tongue, oesophagus, and fore-omach, but practically absent from the glandular stomach, duodenum, ileum, and ectum. In the other species, mast-cells were numerous throughout the whole of the igestive tract, presenting a large individual variation. In the tongue and oesophagus ast-cells were found mainly in the submucosa and lamina propria, where they were cated near the epithelium. In the stomach, mast-cells were more numerous in the ucosa and concentrated in the muscularis mucosae and between the necks of the ands. In the duodenum, mast-cells were also more numerous in the mucosa, being ncentrated in the muscularis mucosae and frequently also in the summit of the villi. the ileum and rectum mast-cells were less numerous than in the stomach and uodenum, keeping the same disposition as in these. The contribution of the mast-lls to the histamine content of the digestive tract is discussed.

Introduction

MAST-CELLS are present in the digestive tract of all classes of vertebrates (Michels, 1938). Although these cells have been thoroughly udied by Michels (1923) and by Bolton (1933) in the wall of the alimentary anal of lower vertebrates, we could not find any report on their distribution the digestive tract of laboratory animals. It is known that the wall of the igestive tract has a high content of histamine, whose cellular location has not een ascertained. Since Riley and West (1953) have demonstrated a striking rrelation between the mast-cell population of a tissue and its content in istamine, it seemed to us interesting to study the mast-cell distribution roughout the digestive tract of some laboratory animals. In the present aper we report our results.

Material and Methods

The digestive tract, including the tongue, of ten rats, six dogs, six cats, and ight guinea-pigs, was studied. The animals were adults of both sexes. They ere bled under ether or nembutal anaesthesia and the digestive tract imme-iately removed. The tongue was fixed by perfusion of the fixative through the essels, and the whole tract, from oesophagus to rectum, was filled with the xative. All the tissues were then immersed in it for 20 to 24 hours. As a xative a 1% solution of lead sub-acetate in 50% ethanol with 0·5% of acetic cid was used. This mixture has been found to be the best fixative for mast-cells

Quarterly Journal of Microscopical Science, Vol. 97, part 2, pp. 251–256, June 1956.]

(Ferri and Mota, 1955). Mast-cell distribution was studied in the tongue, oesophagus, cardiac, fundic, and pyloric regions of the stomach, duodenum, ileum, and rectum, in frozen sections 50μ thick, stained by a 1% toluidine blue solution adjusted to about pH 4 with acetic acid. Sections were mounted in Technicon mounting medium.

Results

Rat. In the digestive tract of the rat mast-cells were found to be extremely numerous in the tongue (fig. 1, A), plentiful in the oesophagus (fig. 1, B), present in small numbers in the fore-stomach (fig. 1, C), very scarce in the submucosa of the fundic (fig. 1, D) and pyloric regions, and practically absent from the duodenum (fig. 1, E), ileum, and rectum. In the tongue these cells were found in the connective tissue between the muscle-bundles and in the lamina propria of the mucosa. In the oesophagus the mast-cells were located mainly in the mucosa and a few in the tunica muscularis. In the fore-stomach and in the fundic region they were found in the submucosa almost exclusively. On the other hand, the digestive tract of the rat from stomach to rectum is rich in eosinophils, which are distributed in all layers and, like the mast-cells in other species, are located in the lamina propria of the mucosa and concentrated in the muscularis mucosae.

Guinea-pig, dog, and cat. In these species the mast-cell distribution was very alike and they will be described together. However, in the cat and dog mast-cells seemed to be more numerous than in the guinea-pig. Unlike the rat, in these species, mast-cells were present in all segments of the digestive tract, but their number was quite variable, in some animals being very high, while scarce in others. In the tongue the mast-cells had the same distribution as in the rat, but seemed to be less numerous (fig. 1, F). Frequently these cells were found in the connective core of the secondary papillae near the epithelium. This occurred mainly at the dorsal surface of the tongue of the guinea-pig.

In the oesophagus the mast-cells were found for the most part in the submucosa and in the lamina propria and in small numbers in the muscular layer. In the lamina propria mast-cells were found near the epithelium in the connective tissue of the papillae (fig. 1, G), this fact being more evident here than in the tongue. In the dog there were also many mast-cells around the acini of the oesophageal glands.

Fig. 1 (plate). Photomicrographs of frozen sections after fixation in lead subacetate / ethanol / acetic acid and staining in toluidine blue. Nuclei stain lightly, so that practically every black dot is a mast-cell. The scales represent 20μ.

A, tongue of rat; numerous mast-cells between the muscle-bundles.

B, oesophagus of rat; note mast-cells in the submucosa.

C and D, fore-stomach and glandular stomach (fundic region) of rat; few mast-cells can be seen in the submucosa.

E, duodenum of rat; no mast-cells are observed in the field.

F, tongue of cat; mast-cells in the connective tissue of the papilla.

G, oesophagus of guinea-pig; observe mast-cells in the lamina propria near the epithelium.

H and I, stomachs of guinea-pig and dog; note numerous mast-cells concentrated in the mucosa, while only a few can be seen in the submucosa.

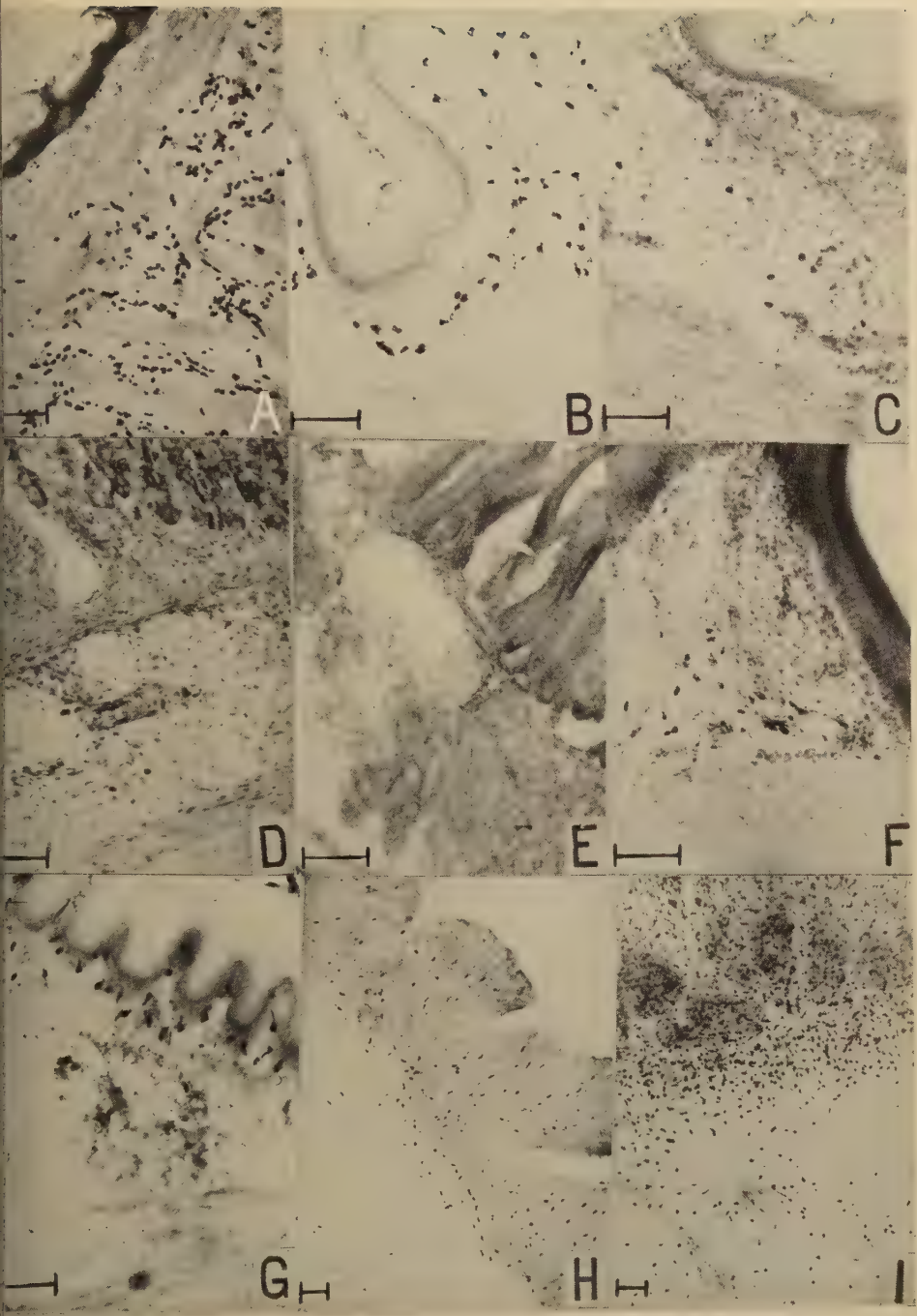

FIG. 1

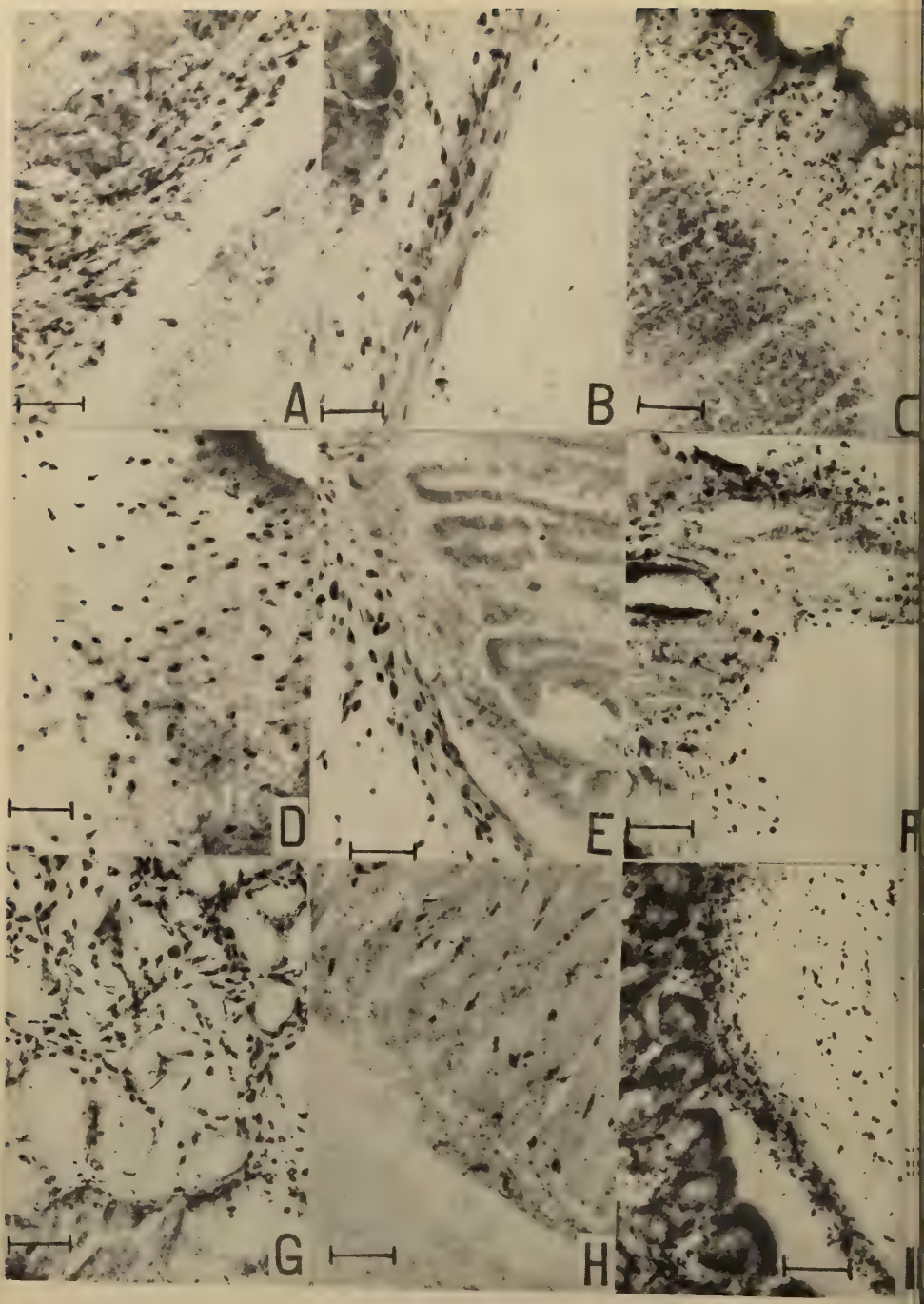

FIG. 2

In the stomach, in all three regions the mast-cells were more numerous in he mucosa than in any other layer (fig. 1, H and I), although the mast-cell opulation of the mucosa showed a great deal of individual variation. Mast-ells were characteristically concentrated in the muscularis mucosae (fig. 2, A nd B) or between it and the bottom of the glands, and were frequently located etween the necks of the glands (fig. 2, C). Mast-cells were found very near the pithelium, and the nearer the lumen the more cells were found with only a ew granules that had frequently lost their metachromasia. These cells re-alled the 'atypical mast cells' of several authors (Michels, 1938, p. 306). No ifference in the mast-cell distribution was observed between the cardiac, undic, and pyloric region of the stomach, although they seemed to be more umerous in the fundic region (fig. 2, D). In the submucosa as well as in the uscularis externa, there were also many mast-cells. In the latter they were ocated along the muscle-fibres.

The duodenum also was very rich in mast-cells and, in the cat and dog, ometimes much richer than the stomach. Most of the mast-cells were located the mucosa and concentrated in the muscularis mucosae (fig. 2, E). Mast-ells were also scattered through the connective core of the villi, being fre-uently located at the summit of the villi (fig. 2, F), very close to the lumen of he gut. Here also the mast-cells near the surface of the mucosa showed few ranules and loss of metachromasia.

In the dog and cat, in the places where the muscularis mucosae divides into undles that turn around the acini of the Brunner's glands, the mast-cells ere found between these glands (fig. 2, G). The mast-cells were also requently found in the submucosa and in the muscularis externa.

In the ileum the mast-cells kept the same location as in the duodenum but ere less numerous. Most of these cells were located in the mucosa and cattered through the connective core of the villi (fig. 2, H). In the guinea-pig he number of these cells was quite variable, in some animals the lamina ropria of the mucosa being very rich, while in others very poor. In the mphatic nodes or Peyer's patches of the dog and cat, mast-cells were either bsent or few in number.

In the rectum mast-cells were less numerous than in the duodenum and eum. They were present in the connective tissue of the lamina propria, where

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FIG. 2 (plate). Photomicrographs of frozen sections after fixation in lead subacetate / ethanol / etic acid and staining in toluidine blue. The scales represent 20 μ.

A, stomach of dog (fundic region); observe the mast-cells in the muscularis mucosae which, gether with the mucosa, was artificially detached from the submucosa.

B, stomach of dog (pyloric region); observe mast-cells concentrated in the muscularis ucosae.

C, stomach of cat (pyloric region); mast-cells between the necks of the glands.

D, stomach of guinea-pig (fundic region); mast-cells scattered in the mucosa.

E, duodenum of dog; observe mast-cells concentrated in the muscularis mucosae.

F, duodenum of dog; mast-cells in the summit of a villus.

G, duodenum of dog; mast-cells between the Brunner's glands.

H, ileum of guinea-pig; mast-cells scattered in the connective tissue of the villi.

I, rectum of dog; observe mast-cells mainly in the submucosa and in the muscularis mucosae.

frequently they were concentrated in the muscularis mucosae (fig. 2, 1). They appeared in the submucosa, mainly around its vessels and were scattered through the muscle fibres in the muscularis externa.

General remarks. Some general features were common to the species observed with the exception of the rat. These were the large numerical variation of the mast-cells from one animal to another, the great richness of the mucosa in mast-cells, the peculiar distribution of these cells concentrated in the muscularis mucosae and between it and the bottom of the glands, and their gradual diminution from the duodenum to the rectum.

Discussion

The characteristic distribution of the mast-cells throughout the digestive tract with their concentration in the mucosa seems to parallel the findings in lower vertebrates of Bolton (1933) on mast-cell distribution in the alimentary canal of salmonoid fishes. Bolton's figures are very clear.

Recently, while this paper was being prepared, Arvy and Quivy (1955) reported on the mast-cell distribution in the digestive tract of the dog. They obtained results similar to ours.

Since recent data have suggested a relation between the mast-cells and the histamine content of the tissues, it is interesting to compare the distribution of the mast-cells in the wall of the digestive tract with its histamine distribution. The wall of the digestive tract, mainly that of the stomach and duodenum, has a high content of histamine, whose cellular location and possible function are not known (Feldberg, 1954). Histamine has been chemically isolated from the gastric and intestinal mucosa of several species (Bayer and Dale, 1911; Abel and Kubota, 1919; Gerard, 1922; Sacks, Ivy, Burgess, and Vandolah, 1932).

Quantitative estimates by biological assay of the histamine content of the wall of the stomach and gut have been reported by several authors (Gavin, McHenry, and Wilson, 1933; Gaddum and Schild, 1934; Schild, 1939; Emmelin and Kahlson, 1944; Trach, Code, and Wangersteen, 1944; Douglas, Feldberg, Paton, and Schachter, 1951). These authors have shown that the histamine content of the digestive tract is very variable from one animal to another and that its concentration is low in the oesophagus but high in the stomach and duodenum, whence it gradually decreases to the rectum. Throughout the whole of the digestive tract, from the stomach onwards, the histamine concentration is higher in the mucosa than in the other layers, while the submucosa contains more histamine than the muscularis externa. Particularly in the digestive tract of the dog the distribution of the histamine has been carefully studied by Douglas and others (1951). In the stomach and small intestine of the dog, where the muscularis mucosae could be separated from the remainder of the mucosa, most of the histamine was usually found in the former. The cellular location of the mucosal histamine was studied by Feldberg and Harris (1953) by the Linderstrøm–Lang procedure. They found in the mucosa of the stomach two regions of higher histamine content: the

region of the parietal cells and that of the muscularis mucosae. In the duodenum they also found two levels of maximal histamine content, one near the lumen in the region of the villi, and another in the muscularis mucosae. Perhaps these authors did not observe the mast-cells because they used formaldehyde fixation, which preserves these cells badly (Ferri and Mota, 1955).

There is a strong parallelism between the histamine and mast-cell distribution along the whole of the digestive tract, and also a great individual variation in both. There is a relatively small quantity of both in the oesophagus and a high quantity in the stomach and duodenum, whence there is a gradual decrease in both up to the rectum. They are mainly located in the mucosa and in it they are usually concentrated in the muscularis mucosae. In the same way, the high histamine concentration obtained by Feldberg and Harris (1953) in the region of the parietal cells of the gastric mucosa and near the lumen in the region of the duodenal villi could also be explained by the mast-cell concentration in these regions. However, to this parallelism there is one exception, that of the digestive tract of the rat, in which the high histamine content from the glandular stomach to the rectum is not paralleled by the occurrence of large number of mast-cells (Mota, Beraldo, Ferri, and Junqueira, 1955). Thus it is possible that in the other species studied not all the histamine of the digestive tract is located in the mast-cells. Therefore, although the parallelism between histamine and mast-cells does not prove that all the histamine of the digestive tract of the cat, dog, and guinea-pig is located in the mast-cells, it strongly suggests that a part at least of the histamine is located in them. However, compound 48/80, which promotes a vigorous extrusion of mast-cell granules (Mota, Beraldo, and Junqueira, 1953) and has a strong depleting effect on the histamine bound to the mast-cells of the rat (Mota, Beraldo, Ferri, and Junqueira, 1955), has only a very small or no effect on the histamine of the digestive tract of the cat and guinea-pig (Smith, 1953), although their digestive tracts are rich in mast-cells. This fact may be explained by species differences, since the mast-cell of the guinea-pig is very resistant to the action of compound 48/80 (Mota and Vugman, unpublished data).

An interesting possibility arises from the finding of mast-cells in close proximity to the parietal-cells in the stomach. If, as seems probable, they secrete histamine, it could be speculated that this could serve as a stimulus to secretion by the parietal-cell, without a detectable rise in the plasma histamine level.

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The Origin of the Spermatophoric Mass of the Sand Crab, *Hippa pacifica*

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Summary

1. In *Hippa pacifica*, mitotic division of germinal epithelial cells produces primary spermatocytes which, as sustentacular-like cells appear, divide meiotically to form spermatids.

2. Concurrently as spermatids metamorphose into spermatozoa, germinal epithelial cells renew their activity and again produce primary spermatocytes which, as they fill the sacculus, expel the spermatozoa.

3. A continuous spermatogenic mass enters the vas deferens, where an enveloping secretion from the epithelial cells bounding the lumen forms a thin sheath to the sperm mass.

4. The lumen (viewed in cross-section) becomes keyhole-shaped and a secretion from a wedge-shaped group of cells bounded by two deep crypts both surrounds the sheathed sperm mass and forms a ribbon-like supporting stalk and a broad, basal foot.

5. As the lumen again becomes circular (in cross-section), a secretion from the epithelial cells surrounds the completed spermatophore and forms the homogeneous matrix.

6. The spermatophore of *H. pacifica* is both macruran-like, because of its continuous, highly convoluted sperm mass, and anomuran-like, because of its raised spermatophore and broad foot.

7. Neither the anomuran-like spermatophore of a macruran, *Parrabicus antarcticus*, nor the macruran-like spermatophore of an anomuran, *Hippa pacifica*, justifies the inference that these two animals occupy a systematic position intermediate between the Macrura and the Anomura, but rather illustrates the need for consideration of many characters before postulating relationships.

Introduction

THE purpose of this paper on the origin and development of the spermatophoric mass of *Hippa pacifica* is twofold: (1) to extend our knowledge of hippid biology, and (2) to show that in *H. pacifica* the spermatophores are intermediate between the non-pedunculate macruran and the pedunculate anomuran spermatophores.

With the exception of the Cape crayfish (*Jasus lalandii*, see von Bonde, 1936) and the rock lobster (*Parribacus antarcticus*, see Matthews, 1954*a*), all recorded observations indicate that the palinurids, the astacids, the homarids, and the nephropsids produce typical macruran spermatophores consisting of highly convoluted tubes embedded in sticky, putty-like matrices (*Panulirus interruptus*, see Allen, 1916; Fasten, 1917; Wilson, 1948; *P. argus*, Crawford and DeSmidt, 1923; *P. penicillatus*, Matthews, 1954*c*; *Potamobius trowbridgii*, Andrews, 1931; *Homarus americanus*, Herrick, 1895; *Enoplometopus occidentalis*, Matthews, 1954*b*).

With the exception of the hippids, all recorded observations indicate that

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the galatheids, pagurids, and porcellanids produce typical anomuran spermatophores in which the continuous, convoluted tube of the macrurans is broken into distinct ampullae, elevated on peduncles attached to a foot-like base (Galatheida, Paguridae, Porcellanidae, see Mouchet, 1930–1; Paguridae, Matthews, 1953).

On the basis of anatomical characteristics, the families of the tribe Macrura are usually considered more closely related than are the families of the tribe Anomura. Since an anomuran-like spermatophore has been reported for a member of the Macrura (see papers cited by Matthews, 1954*a*), one might expect that a macruran-like spermatophore would ultimately be reported for a member of the more heterogeneous Anomura. This study reports such a spermatophore and points the need for the consideration of both morphological and physiological characteristics in postulating taxonomic relationships between these groups of animals.

Methods and Techniques

Specimens of *Hippa pacifica* (Dana) taken in the vicinity of Waimanalo Bay, Oahu, during the high surf of June 1954, were used in this study. Both males and females were taken. The ratio of males to females was 1:15. Of these, nearly all females were in the berried condition and all males over 1·5 cm long were sexually mature. The reproductive systems of non-anaesthetized males immersed in sea water were removed with the aid of a dissecting microscope.

The testes and right vasa deferentia were either fixed in Bouin's fluid, washed in alcohol (70%), cleared in dioxane, embedded in Tissuemat (54–56° C), sectioned at 10μ, and stained in standard alum haematoxylin and eosin; or they were fixed in Champy's fluid, washed in running water (24 hours), dehydrated in alcohol, cleared in cedarwood oil, infiltrated with xylene, embedded in Tissuemat (54–56° C), sectioned at 4–6μ, and stained in iron haematoxylin.

The left vasa deferentia were immersed for 10 to 30 minutes in an aqueous solution of toluidin blue (1:10,000) and teased open in sea water, and their vitally stained contents studied and drawn.

Observations

The morphology of the testes and the vasa deferentia

The mature testes freed of overlying tissues resemble an elongate H, the right and left sides being formed of whitish, sacculate tubes joined one to the other by a short transverse bridge (fig. 1, *c*). The anterior portion of the testis (*a*) exceeds the posterior portion (*d*) both in diameter and length, although the sacculi (*b*) in all regions are of approximately the same size (0·2 mm).

The proximal portion of the vas deferens (*e*) arises medially at the junction of the posterior horn of the testis and the transverse bridge. This rather thin, funnel-like portion gradually tapers into a minute, straight tube (*f*), which lies contiguous with the intestine. Posteriorly, this minute portion terminates

in two or three tightly coiled loops (*g*) demarcating the anterior boundary of the enlarged vas deferens (*h*). This portion of the vas deferens terminates on the coxopodite of the small, chelate fifth pereiopod (fig. 2, *a*) in a peculiar cup-like projection (*b*), to which completed spermatophores often adhere.

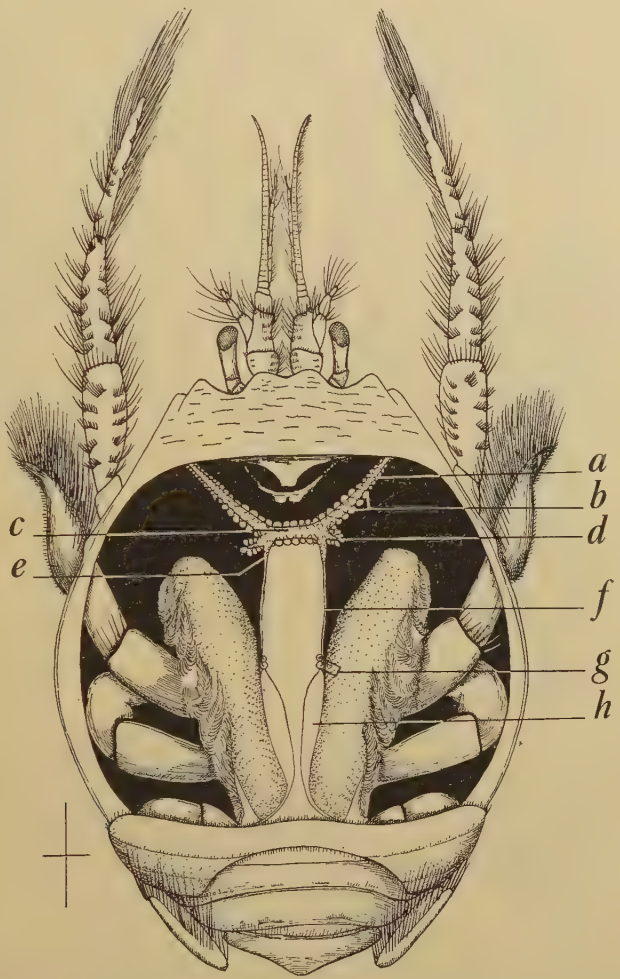

FIG. 1. Dorsal view of dissected *H. pacifica* showing *a*, anterior portion of testis; *b*, sacculi; *c*, transverse bridge; *d*, posterior portion of testis; *e*, funnel-shaped portion of proximal vas deferens; *f*, minute, straight portion of vas deferens; *g*, tightly coiled loops of vas deferens; *h*, enlarged portion of vas deferens. Magnifications as indicated.

The histology of the testes and the vasa deferentia

In histological cross-sections taken through the various portions of the testis (fig. 1, *a*, *c*, *d*), sacculi (*b*) in all stages of maturity are observed. Because the process of maturation in the sacculi of *H. pacifica* so nearly approximates the process of maturation in the sacculi of a nephropsid lobster (see Matthews,

1954*b*, figs. 2, 3, 4, pp. 116–17), only a brief summary is here given. The immature sacculus of *H. pacifica* discloses large primary spermatocytes indistinguishable from the germinal epithelial cells. As the sacculus matures,

FIG. 2. Ventral view of *H. pacifica* showing *a*, small, chelate, fifth pereiopod; *b* (in enlarged insert) cup-like projection.

sustentacular-like cells with indistinct cytoplasm but strongly chromophil nuclei appear. These cells, which lie indiscriminately among the primary spermatocytes, increase in number, but both their origin and method of multiplication remain obscure. It was not ascertained whether the presence of these unique cells influences spermatogenesis, but the study of many sections reveals that as the number of these sustentacular-like cells increases, the number of secondary spermatocytes increases. Then these secondary spermatocytes divide to form spermatids.

Sections through more mature sacculi reveal that, concurrently with the metamorphosing of spermatids, germinal epithelial cells renew their activity and again produce primary spermatocytes; these, as they fill the sacculi, expel the metamorphosing spermatids.

Whereas cross-sections taken from various regions of the testis (fig. 1, *a*, *c*, *d*) are histologically similar, cross-sections taken from various regions of the vas deferens (fig. 1, *e*, *f*, *h*) are histologically different (figs. 3–15). These differences are, for the most part, due to changes in thickness of the epithelial and muscular layers, which in turn affect the size and shape of the lumina.

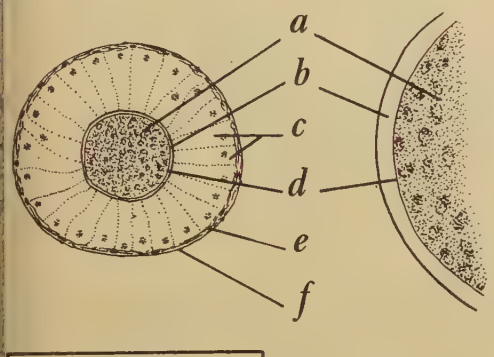

FIG. 3

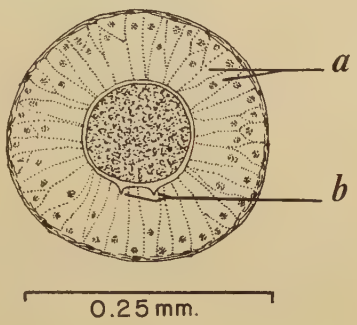

FIG. 4

FIG. 3. Cross-section through the straight, proximal portion of the vas deferens showing *a*, sperm mass; *b*, lumen; *c*, epithelial cells; *d*, sheath of sperm mass; *e*, muscle layer; *f*, connective tissue layer.

FIG. 4. Cross-section through the proximal portion of the vas deferens showing *a*, epithelial cells; *b*, crypts.

Thus the lumen is a gradually changing die through which the sperm mass from the testis is moulded.

The sperm mass (fig. 3, *a*) enters the almost circular lumen (*b*) of the straight, proximal portion of the vas deferens. The lumen here is bounded by epithelial cells (*c*) whose secretion both permeates the loosely packed sperm mass and forms an extremely thin sheath to the sperm mass (*d*). The mechanism by which the sheathed sperm mass traverses the proximal portion of the vas deferens is obscure. Since the muscle layer (*e*) which lies between the epithelial (*c*) and connective tissue layers (*f*) is here poorly developed, it is doubtful if it serves either to mould the sperm mass in conformity to the lumen, or by peristalsis to force the sperm mass along. Both processes are probably accomplished in this region of the vas deferens by the continuous flow of sperm mass from the testis.

Gradually, in more distal cross-sections (fig. 4), the epithelial cells (*a*) lengthen, and two minute crypts (*b*) appear. These crypts deepen (fig. 5, *a*) and demarcate a wedge-shaped group of cells (fig. 6, *a*). Even before this wedge-shaped structure appears, its future position is indicated by a small

group of cells whose cytoplasm has an affinity for the nuclear stain, whereas the cytoplasm of other epithelial cells bounding the lumen retain their affinity

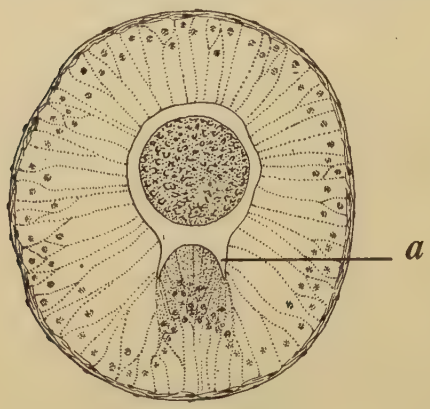

FIG. 5

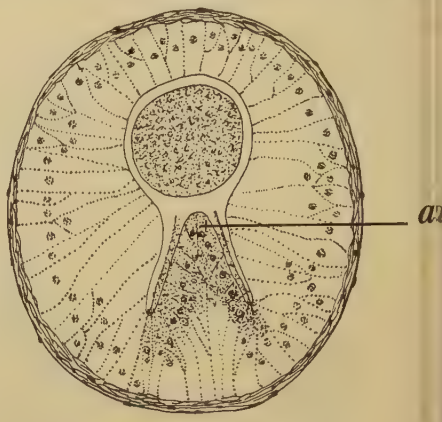

FIG. 6

FIG. 5. Cross-section through the proximal portion of the vas deferens showing *a*, deep crypts.
FIG. 6. Cross-section through the proximal portion of the vas deferens showing *a*, wedge-shaped group of cells demarked by deep crypts.

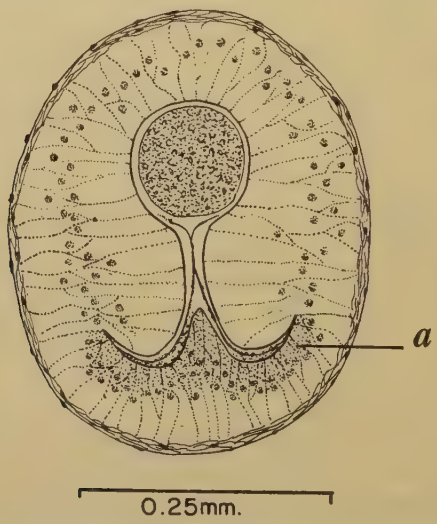

FIG. 7. Cross-section through the proximal portion of the enlarged vas deferens showing *a*, epithelial cells adjacent to wedge-shape structure.

for the cytoplasmic stain. At first, this difference in staining reaction is limited to those cells actually comprising the wedge-shaped structure, but later, as the crypts spread peripherally forming a broad W (fig. 7), the cytoplasm of cells adjacent to the wedge-shaped structure also takes up the nuclear stain (*a*)

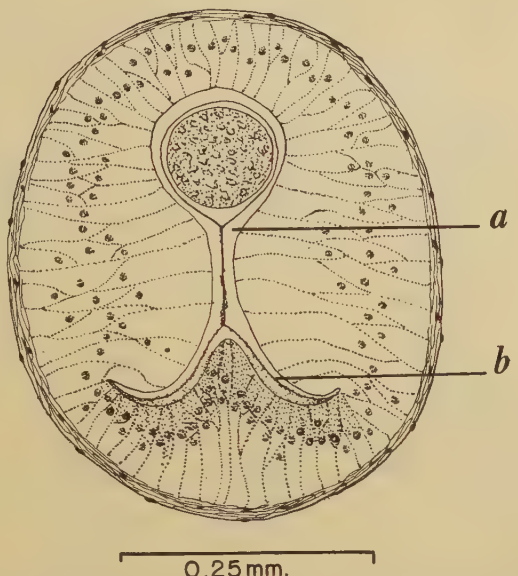

FIG. 8. Cross-section through the proximal portion of the enlarged vas deferens showing *a*, keyhole-shaped lumen; *b*, secretion from epithelial cells bordering W-shaped crypt.

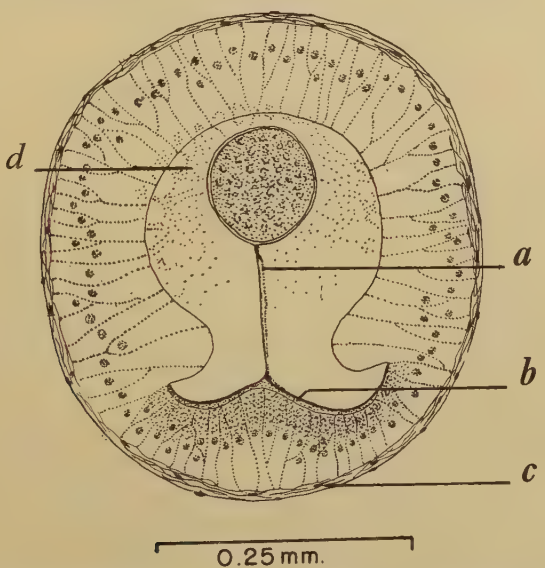

FIG. 9. Cross-section through the proximal portion of the enlarged vas deferens showing *a*, secretion from epithelial cells bordering W-shaped crypt forming ribbon-like stalk; *b*, foot; *c*, muscle layer; *d*, matrix.

By this time a secretion (fig. 8, *b*) from the epithelial cells bordering the crypt fills the keyhole-shaped lumen (*a*) and surrounds the already sheathed sperm mass. Gradually (fig. 9) this secretion forms the ribbon-like stalk (*a*) and the

broad, basal foot (*b*). An increase in thickness of the muscular layer (*c*) suggests the possibility of muscular propulsion of the developing spermatophore although in dissected living specimens no contractile waves of the vas deferens are observed. Still another secretion (*d*) from the epithelial cells bounding the lumen surrounds the sheathed spermatophore and forms the matrix.

In cross-sections through more distal regions of the enlarged vas deferens (figs. 10, 11) both the epithelial cells (*c*) whose secretion forms the ribbon-like stalk and broad, basal foot, and the epithelial cells (*b*) whose secretion

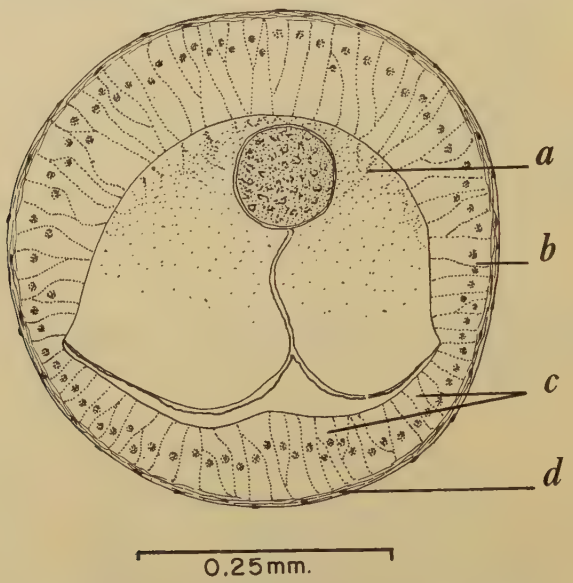

FIG. 10. Cross-section through the enlarged portion of the vas deferens showing *a*, matrix in lumen; *b*, matrix-secreting cells; *c*, ribbon-and-foot-secreting cells; *d*, muscle layer.

forms the enveloping matrix (*a*), gradually decrease in size. Since, however the epithelial cells which lie contiguous with the foot diminish at a more rapid rate, the lumen in cross-section loses its keyhole shape and again gradually becomes more circular.

As both matrix-forming epithelial cells (fig. 12, *b*) and the foot-forming epithelial cells (*c*) diminish, the broad, basal foot (*e*) becomes widely separated from the epithelial cells (*c*). Whether this is the result of still another secretion from the foot-forming epithelial cells remains undetermined; however, a uniform matrix-like secretion now appears below as well as above the foot. Although the foot appears to be in contact laterally with the foot-forming epithelium, more distal cross-sections (figs. 13, *a*) reveal it to be free. The possibility therefore remains that, rather than another secretion, the original matrix now completely surrounds the completed spermatophore.

Since the distal portion of the vas deferens (fig. 1, *h*) is spindle-shaped, in traversing this region the completed spermatophore is crowded and the ribbon

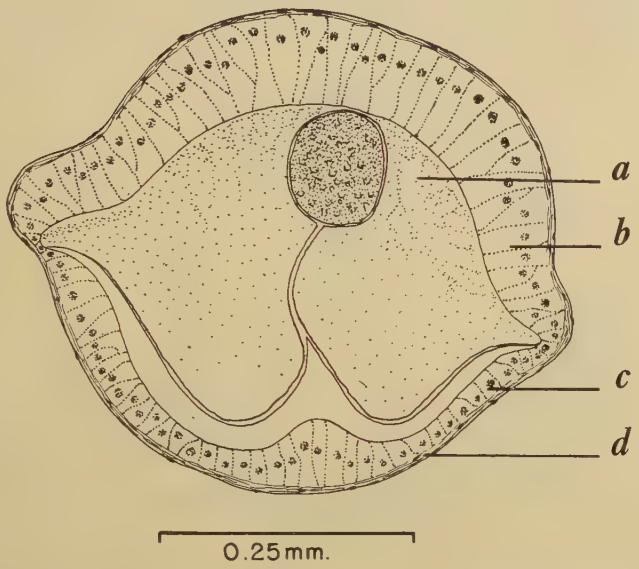

FIG. 11. Cross-section through the enlarged portion of the vas deferens showing *a*, matrix in lumen; *b*, matrix-secreting cells diminishing; *c*, ribbon-and-foot-secreting cells diminishing; *d*, muscle layer.

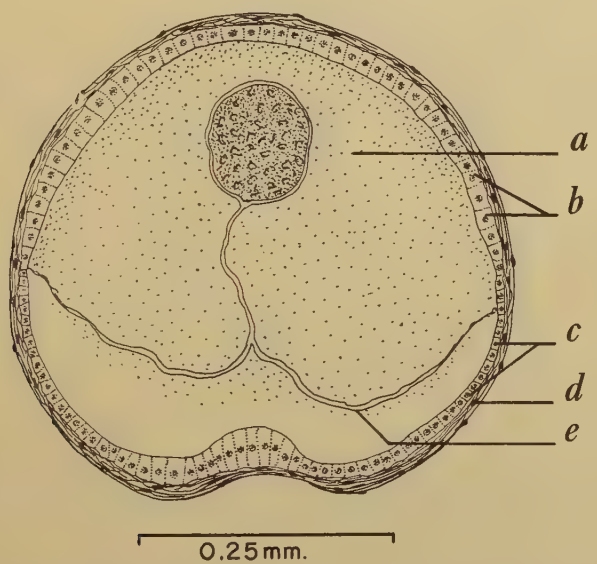

FIG. 12. Cross-section through the enlarged portion of the vas deferens showing *a*, matrix; *b*, matrix-forming cells; *c*, foot-forming cells; *d*, muscle layer; *e*, foot widely separated from foot-forming cells (*c*).

(fig. 14, *a*) is bent. The epithelium (*b*) is now an extremely small layer of cells bounding, in cross-section, an almost completely circular lumen (*c*). The circular and longitudinal muscular layers (*d*) are here well developed. The

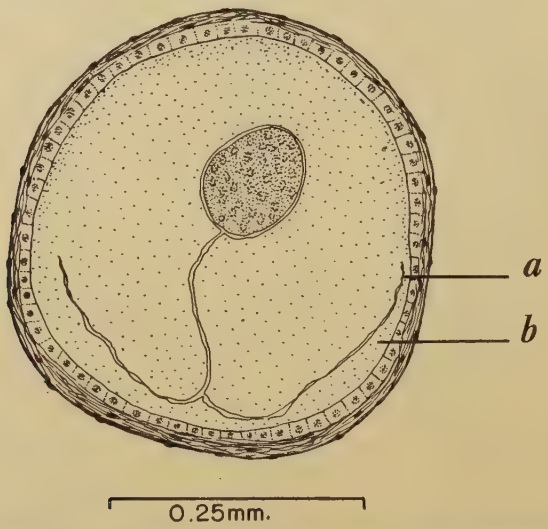

FIG. 13. Cross-section through distal portion of the vas deferens showing *a*, foot, free from foot-forming cells; *b*, matrix.

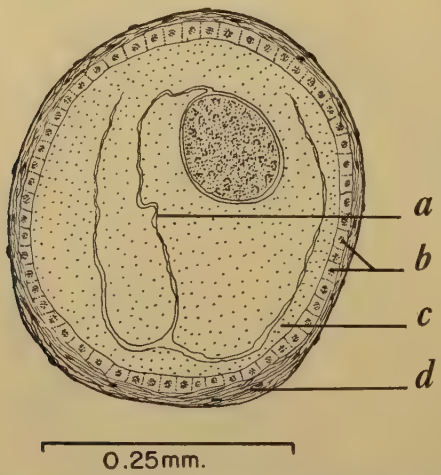

FIG. 14. Cross-section through distal portion of the vas deferens showing *a*, bent ribbon; *b*, epithelial cell layer; *c*, circular lumen; *d*, muscle layers.

latter are probably used during the actual process of spermatophore deposition.

Fig. 15 is a drawing made from a portion of the distal region of the vas deferens stained with toluidin blue and dissected to show the contained spermatophore. The encompassing matrix, which otherwise would conceal

he spermatophore, is dissolved by weak KOH. Whereas the study of cross-ections may leave some doubt as to whether the spermatophore is continuous, he study of dissected, vitally stained portions of the vas deferens leaves no oubt that the spermatophore of *H. pacifica* is a continuous, highly con-oluted tube (*a*) raised by a continuous, ribbon-like stalk (*b*), attached to a road foot (*c*).

Since I have not observed the attached spermatophore in nature, I cannot ay whether the foot flattens or remains curved upward. This much can be aid with certainty, it is the foot and ribbon which are brittle and offer sup-

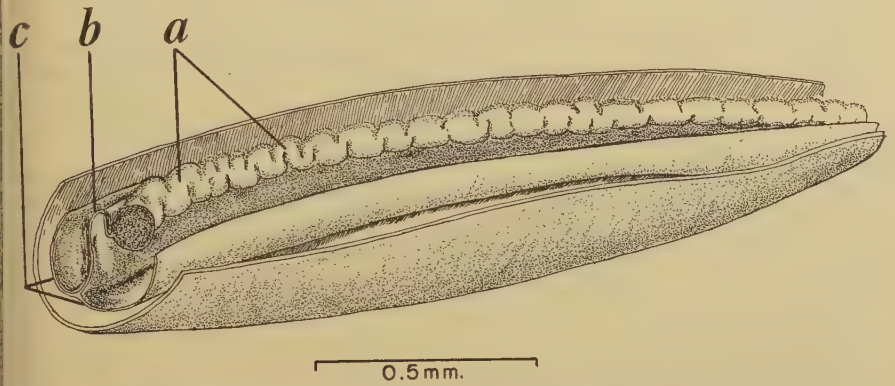

Fig. 15. Distal portion of vas deferens stained with toluidin blue and dissected showing *a*, convoluted sperm tube; *b*, ribbon-like stalk; *c*, broad foot.

port; the matrix, when the spermatophore is forcibly removed from the living animal, is soft and probably serves as the adhesive substance. Hardening, but no colour change of the matrix is noted. The nature of the completed sperma-tophore suggests mechanical liberation of spermatozoa.

Discussion

Although the study of macruran spermatophores indicates that the funda-mental plan is a highly coiled spermatophoric tube embedded in a homo-geneous putty-like matrix, nevertheless, as previously stated, an anomuran-like spermatophore has been described for a member of the Macrura (*Parri-bacus antarcticus*, see papers quoted by Matthews, 1954*a*). This disclosure alone has little meaning except, perhaps, to point out that factors effecting change are not uniformly expressed in all organ-systems of even closely related animals. This applies to both the Anomura and the Macrura. Recent studies (in press) on the spermatophores of the land crabs *Coenobita* and *Birgus* illustrate the same point. These animals, although remarkedly adapted for a terrestrial habitat, still rely on a reproductive system keyed to an aquatic (marine) environment.

Although the study of anomuran spermatophores indicates that their basic plan is a discontinuous spermatophoric tube (with ampullae of sperm) raised on slender stalks attached to a base-like foot, variations on this plan should be

expected in such a diverse group. That *H. pacifica* produces a spermatophor midway between those of the Macrura and the Anomura is, when taken b itself, of no great significance. It merely serves to illustrate that when more learned concerning both the anatomy and physiology of organ-systems, man old-established lines of relationship may perhaps be cut across. At the preser state of our knowledge concerning *H. pacifica* it is wrong to refer to thi animal as crab-like, solely because of the development and position of it abdomen; and it is equally wrong to refer to it as lobster-like, solely becaus of the macruran nature of its spermatophore.

This paper forms Contribution No. 80 of the Hawaii Marine Laboratory.

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Colloidal Properties of the Mesogloea in Species of *Leucosolenia*

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With one plate (fig. 1)

Summary

Oscular tubes rapidly collapse and disperse in isotonic potassium nitrate solution, the cells dissociating and rounding off, and the mesogloea softening and swelling. Reasons are given for regarding the action as a direct one on the mesogloea and intercellular 'cement'.

Transference of the collapsing tubes to sea-water, or isotonic calcium chloride, results in an immediate stiffening of the mesogloea, and the swollen cells form characteristic processes which are best seen after the cells have been in the nitrate solution for an optimum period depending on the temperature. They are not formed after treatment with M/100 potassium cyanide.

The action of other isotonic salt solutions is briefly described. When a comparison is made of the times taken for the tubes to become plastic, the ions respectively used fall into three series which are the same as those found in regard to the dispersiveness of certain hydrophilic organic colloids.

The experiments provide evidence that the mesogloea is secreted by the choanocytes or by the amoebocytes close beneath them, and that the secretion stiffens into a firmer gel as it passes into the spicule zone. The degree of firmness also varies apparently according to the health of the tube.

The action of distilled water and acidified sea-water are described. After the spicules have dissolved the tube still retains its shape and much of its support can thus derive from the mesogloea. The relative functions of the mesogloea and the spicules are briefly discussed.

Contents

Introduction

REFERENCE has already been made (Jones, 1955, *a* and *b*) to the disruptive action of 5% potassium nitrate solution on the oscular tubes of *Leucosolenia complicata*, but only the corrosion of the spicules was described in detail. In this paper an account of the action on the rest of the sponge will

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be presented, together with the action of some other isotonic salt solutions since they reveal some interesting properties of the cells and mesogloea. Before describing the phenomena, however, some preliminary remarks on the physical properties of the mesogloea will be given.

Properties of the mesogloea

The mesogloea in the calcareous sponges consists of a hyaline ground substance (Minchin, 1900), which, from its staining reactions, is probably composed of collagen (Herlant-Meewis, 1948*a*). In *Clathrina coriacea* it contains rows of small granules representing a network of very fine elastine fibrils (Herlant-Meewis, 1948*a*). These, however, have not been seen by the present author in sections of *L. complicata*, in which orcein appears to stain only the cell-processes, spicules sheaths, and the crinkled epidermal layer.

The isotropic nature of the mesogloea is further revealed when living or dead pieces of the wall of *L. variabilis* are rotated between crossed nicols, for no birefringence is detectable between the spicules. The spicules themselves appear equally distinct at all angles of rotation when the analyser nicol is withdrawn.

After brushing the choanocytes away from pieces of the wall of *L. complicata* or *L. variabilis*, the spicules remain firmly embedded in what appears to be a clear membrane. Prodding the gastral rays of the quadriradiates or the embedded spicule rays reveals the firmness with which they are held. The wall itself seems tough and inelastic when attempts are made to stretch it with a pair of needles, and stronger efforts tear the piece into two. The torn edges then exhibit projecting spicule rays, of which some still retain adhering calcoblasts, while in between, the wall substance is retracted. No projecting filaments or jagged edges are visible and there is no sign of a fluid seeping from the wound. The observations suggest that the spicules are not merely anchored by the processes of the calcoblasts on the tips of the rays, but are maintained in position by the firm mesogloea; and further evidence for the firmness of this substance is afforded by the young quadriradiates, for these bulge out the choanoderm against the spongocoelic water pressure instead of sinking farther into the mesogloea as they develop (Jones, 1954*b*). However, the osculum can constrict, as when stimulated by bright light, and the whole tube can change shape, so that the mesogloea must at times be plastic. Schulze (1885) likewise believed that 'the basal substance . . . is sometimes gelatinous, sometimes firm, and sometimes even of cartilaginous hardness', while a similar variation in consistency of the ground substance of *Iotrochota birotulata* has been noticed by de Laubenfels (1932), though here, as with other siliceous sponges (Loisel, 1898), this substance is apparently intracellular.

Materials and Methods

The action of 5% potassium nitrate solution on the oscular tubes was first discovered when washing out the chloride ions from material left unfixed by error, in preparation for silver staining. In later experiments a solution a

0·65 M was used, since this is isotonic with sea-water, according to calculations from data given in the *Handbook of Chemistry and Physics*, 35th edition, 1953–4 (Chemical Rubber Publishing Co.). There is little difference between the two strengths of solution.

The species selected was mainly *L. botryoides* (Minchin, 1904), which in the spring produces many long oscular tubes free from diverticula. Tubes of *L. complicata* have also been used in the same season, but *L. variabilis* is more easily found in the late summer. Tubes of the latter species have been employed on only two occasions.

Two or three oscular tubes excised from the specimen were usually placed in 20 or 30 ml of the solution in a Petri dish, after having been washed for at least 3 minutes in a similar solution. Besides the potassium nitrate, other isotonic solutions were used, and when their effects were being compared it was found best to have the dishes resting on a long glass plate clamped at a height which allowed the microscope stage to be moved along beneath; this facilitated observation without disturbing the material. All the tubes in such experiments were derived from the same specimen and similar in size. The strengths of the isotonic solutions employed were as follows:

Salt	*Molarity*	*pH at start*	*pH after 8 days in dish*
KNO_3	0·65 M	6·5	7·0
LiCl	0·50 M	7·0	7·0
NaCl	0·54 M	6·5	7·5
KCl	0·54 M	7·0	7·0
$MgCl_2$	0·36 M	6·5	7·5
$CaCl_2$	0·36 M	6·5	7·0
K_2SO_4	0·48 M	7·0	7·0

(The pH determinations were made by using B.D.H. Universal Indicator.)

When the action of different salt solutions was being compared, the experiment was performed in a constant temperature room (15° C); most of the other experiments were done at room temperature and the figures given in the text indicate the rough means of the temperature noted during a typical experiment.

Tubes immersed in the potassium nitrate solution and fixed at 15-minute intervals were sectioned at 10 μ by the Peterfi celloidin-paraffin method. A solution containing 2 or 3 drops of 2% osmium tetroxide in 30 ml sea-water was used as the fixative, and the sections were subsequently stained with iron haematoxylin and eosin.

Results

The action of 0·65 M potassium nitrate on oscular tubes

The first events noticed after placing a healthy oscular tube of *L. botryoides* in 0·65 M potassium nitrate (16–23° C) are the cessation of water currents, the closure of the pores, and the gradual contraction of the tube, which

constricts particularly at the oscular rim. Generally the pores have becom distinct after about 10 minutes in the solution, and the process of contractio is completed 2 or 3 minutes later. In a typical case a tube of length 1,450 μ an basal diameter 630 μ contracted to dimensions of 1,360 μ and 560 μ respectively

The tube next becomes progressively softer and less elastic. Thus whe prodded gently with a needle it yields more readily and eventually does no recoil at all. The softening is associated with a swelling of the mesogloea an a separation of the dermal cells, for the tube in optical section now appears t be bordered by a clear zone containing the spicules with, on the outside, a lin of spherical cells derived from the pinacoderm, and on the inside, the brownis zone of swollen collar-cells. Between the spicules in the still firm mesogloe lie other rounded cells, probably representing the porocytes and amoeboi cells of the mesogloea. The spherical pinacocytes are first seen at abou 15 minutes from the start, although the clear zone is more readily observe after half an hour.

At this time (half an hour) the wall has become so soft that the tube canno maintain its cylindrical form and sagging occurs, beginning usually at th oscular end, the upper part sinking and the whole spreading transversely, an to some extent longitudinally, with the flattening of the tube. The spherica cells are carried out as the mesogloea swells and disperses, and they ar eventually set free and move with the currents induced in the water by, fo example, the breath of the observer. The spicules likewise are carried out wards, but tend to settle on the substratum near the collapsed tube, th monaxons generally moving the farthest distance (fig. 1, A).

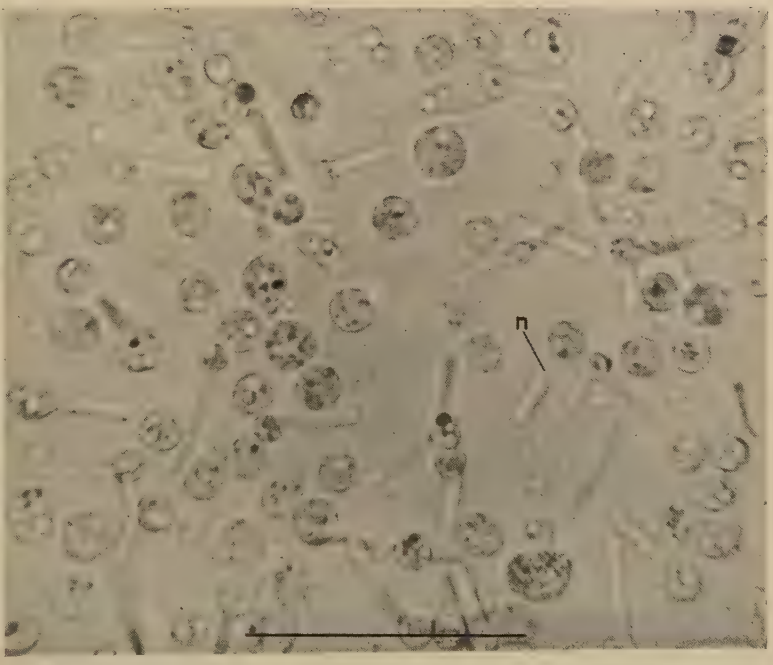
In this condition the mass is very sticky and adheres to the glass beneath the whole is very plastic and the spicules are considerably looser than in th normal tubes and can be moved apart or rotated by means of a needle. Ther is no sign of any filaments lashing the spicules together, and those that hav fallen apart from the collapsed tube appear to be quite naked.

After 2 days all that remains of the tube is a tangle of spicules containing small brownish mass of cell debris (mainly choanocyte remnants) and sur rounded by a litter of partly corroded spicules. No spherical cells are the apparent, and from their exceedingly swollen appearance earlier it is probabl that they have burst; thus after 18 hours their diameters are 10–13 μ, whereas the diameters of most cells teased from an untreated tube are 7–9 μ.

Control tubes placed in a dish of sea-water alongside that containing th specimens in potassium nitrate solution stay healthy for at least a day an maintain a current of water throughout this period; some contraction, bu never collapse, of the tubes occurs.

The spreading process can be markedly hastened once the onset of collaps has been reached by lowering the level of the solution in the dish until it lie beneath the mass. This then spreads rapidly and fairly smoothly in all direc tions, the spicules sliding along with the mesogloea and separating usuall evenly from each other. Here and there, however, spicules are seen to jer apart, when doubtless an interlocking of the spicule rays has allowed th

A



B

FIG. 1. A, part of the surface of a tube of *L. botryoides* which is collapsing after one hour's immersion in a 0·65 M solution of potassium nitrate. The mesogloea has swollen, carrying out dissociated pinacocytes, monaxons, and multi-radiate spicules. The line represents 100 μ.

B, spherical cells with processes obtained by partially dispersing a tube of *L. botryoides* in 0·65 M potassium nitrate solution and then transferring it to sea-water and squashing it on a slide beneath a cover-slip. n, nucleus. The line represents 50 μ.

development of strain which is suddenly released as the spreading continues. The whole mass is very flexible and plastic in this condition and is easily stretched, for example, by suction with a pipette.

Sections of tubes fixed at the half-hour stage reveal definitely that the pinacocytes have rounded off and form the outer zone of spherical cells, while the porocytes are in the process of becoming spherical. The mesogloeal layer is thicker than in the control tubes and contains the spicule rays still with unswollen calcoblasts mounted on them, even after three-quarters of an hour. These cells possess densely staining nuclei, but the nuclei of the pinacocytes tend to be indistinct. The choanocytes have lost their collars, but in many the flagellum is still present (after three-quarters of an hour), together with the blepharoplast, the rhizoplast, and the second siderophil corpuscle (see Duboscq and Tuzet, 1939). In one region the choanocytes were clearly interconnected by swollen cell-extensions midway up their cell-bodies, similar to those described by Duboscq and Tuzet (1939) on the vespiform type of choanocyte in *Sycon raphanus*. Most of the collar-cells, however, contain large vacuoles and appear to be in the course of disintegration.

Some of the effects produced by the potassium nitrate solution can be seen more clearly when pieces of the oscular tubes of *L. complicata* (obtained by bisecting a tube into longitudinal halves) are immersed in the solution with the choanoderm uppermost. The beating of the flagella can then be observed and continues for as long as 30–40 minutes (17·5° C), while spherical cells soon become obvious at all levels of focus in the mesogloea (8 minutes), particularly at the oscular rim. The pieces progressively flatten as the mesogloea softens, and they become flat after half an hour. The collar-cells swell and rise as a single layer with the small, developing spicules attached beneath, but the fully grown spicules usually remain firmly embedded in a transparent mesogloeal 'membrane' for considerably longer. The choanoderm is easily removed from this 'membrane' by means of a brush once the cells have risen, and in one case, in which part of the piece was curled over the rest, the monaxons were firmly held by the 'membrane' for 2 hours, at least $1\frac{1}{2}$ hours after the collar-cells had become level with the tips of the gastral rays. The mesogloea just beneath the choanoderm thus seems to be more rapidly swollen in the potassium nitrate solution than does the mesogloea of the outer spicule zone, though possibly the collar-cells have secreted a slime beneath their bases. However, when aggregates of choanocytes (obtained by brushing pieces of the tube in a watch-glass and leaving for half an hour) were transferred to potassium nitrate solution, the cells merely swelled, remaining closely contiguous with no trace of a slime between them.

Maas (1906) observed that the flagellated cells remain attached together while the dermal cells fall apart when larvae of *Sycandra raphanus* are placed in calcium-free sea-water, and a similar result is obtained with the potassium nitrate solution here, for the dermal cells are separated whereas the collar-cells rise as a coherent layer. However, it is still possible that the collar-cells are 'cemented' together (as Minchin and Reid, 1908, and Robertson and

Minchin, 1910, have suggested) by a colloidal substance similar to the mesogloea, for the swollen collar-cells can be separated by movements of the liquid and they can also pile up on one another like a mass of soap bubbles, as is seen when slices of an oscular tube of *L. botryoides* are set on edge in the potassium nitrate solution. Thus there is only an apparent difference between the pinacocytes and choanocytes, which clearly arises from the differences in form and arrangement of these two types of cell. The pinacocytes are normally stretched and flattened cells, whereas the choanocytes are cylindrical and closely packed side by side, so that the surfaces of the former must separate as the cells round off after dissociation, whilst the latter cells remain contiguous owing to surface tension.

Variations in reaction rate

It has been stated already that the tubes tend to collapse first at the oscular rim when placed in isotonic potassium nitrate solution, and the same is true for the tips of the lateral diverticula. Furthermore, evidence has been given for believing that in *L. complicata* at least there is often a layer of more readily swollen mesogloea adjacent to the choanoderm. Such differences in reaction-time have also been observed between oscular tubes of different, and sometimes even the same, specimens, even when the temperature of the solution was the same. For example, a contracted tube of *L. botryoides* had not flattened after $3\frac{1}{2}$ hours in the nitrate solution, though its monaxons and some spherical cells had fallen out, whereas the healthy, expanded material flattens after about 35 minutes at the same temperature (16° C). Tubes of *L. complicata* have also flattened without the mesogloea swelling as much as normal, even after $2\frac{1}{2}$ hours in the solution, the spherical cells eventually bursting close to the flattened tube. Results have also varied when the tubes have been derived at different times from the same specimen, and when their size has differed considerably. Much would seem to depend on whether the material is actively growing or not, and it is therefore essential when doing comparative experiments to use oscular tubes derived simultaneously from the same specimen and preferably of equal dimensions.

There is little difference between the young, healthy tubes of *L. botryoides* and *L. complicata*, except that the latter tend to collapse a little later than the former; but when tubes of *L. variabilis* were tried, spherical cells were only seen after $3\frac{1}{2}$ hours in the 5% potassium nitrate solution used, while the collapse occurred at 9 hours (18° C). Direct comparison between this species and the others has not been attempted, since they develop at different times of the year, but the considerable difference suggests that the mesogloea of *L. variabilis* consists of a stiffer jelly, at least in the spicule zone, from which the choanoderm readily separates as with pieces of *L. complicata*.

The effect of varying the concentration of the potassium nitrate solution

The rate of the reaction depends upon the concentration of the potassium nitrate solution. For example, three equivalent oscular tubes of the same

pecimen of *L. botryoides* placed respectively in three dishes containing 10%, %, and 2½% solutions, collapsed after 27, 85, and about 200 minutes respectively, and in a similar experiment with *L. complicata* the corresponding times were 50, 155, and over 400 minutes (17° C). Thus the concentration of the potassium nitrate influences the rate of the reaction, which cannot therefore be due merely to the removal of the ions of sea-water, and this is confirmed by the observation that a mixture in equal volumes of sea-water and isotonic potassium nitrate solution causes the partial collapse of the tubes of *L. botryoides*, while sea-water diluted with its own volume or with 3 times its volume of distilled water does not soften equivalent tubes appreciably after a day (16° C). There is otherwise little difference between the three concentrations, except that the cells are more swollen at the time of collapse in the weaker solutions. For example, in the second experiment mentioned above the maximum cell diameters of the choanocytes seen soon after the collapse in the 10%, 5%, and 2½% solutions were respectively 10 μ, 12 μ, and 13 μ. The size of the cells depends on the time they have been in the potassium nitrate solution, and probably on the concentration of the solution.

Transference back to sea-water

The return of the oscular tubes to sea-water from the potassium nitrate solution results in a rapid stiffening of the softened wall without any apparent contraction. Whereas before the tube swayed about when the solution was disturbed, now the whole has become firm and lost its stickiness, while the spicules cannot be separated or rotated as before by means of a needle. The cells, however, remain spherical for at least 7 minutes and remain in position, so that the spicule anchorage is not due to a re-attachment of the calcoblasts to the rays and to each other.

After about 7 minutes in the sea-water the outer spherical cells lose their rounded outline and push out one or more cylindrical processes mainly along the surface of the jelly, though such processes are also formed by the cells within. They usually separate from the rest of the cell after a time.

Transference of the material back to the potassium nitrate solution is followed by the almost immediate reappearance of spherical cells and dispersion of the mesogloea; and the return of the material once more to sea-water results again in its coagulation. Prolonged immersion (18 hours) in the sea-water, however, brings about a softening and disappearance of the mesogloea, either by cellular or bacterial activity, sections having revealed the presence of rows of bacteria in the swollen mesogloea of a tube returned to sea-water before fixation. After about a day in the sea-water the tube remnants display only a tangled mass of spicules surrounded by clusters of cells, isolated cell-processes, and often bacterial slime.

The stiffening action of the sea-water is probably to some extent at least due to the presence of calcium ions, since transference to isotonic calcium chloride, but not to isotonic magnesium, sodium, or potassium chloride, also results in a stiffening almost as marked as when sea-water is used, as estimated

by pushing the tube through the solution with a needle. Calcium ions are known to have an important stabilizing effect on many hydrophilic organic colloids, since their divalency enables them to form links between electronegative groups on adjacent molecules, and Galtsoff (1925) has already found that calcium (and magnesium) ions are necessary for the aggregation of separated dermal cells of *Microciona prolifera*, these ions apparently stabilizing the surface coats of the cells.

Transference of the spherical cells to sea-water

The swelling of the cells in isotonic potassium nitrate solution can be explained by an osmotic uptake of water caused by the entry of K^+ and NO_3^- ions along a concentration gradient, the rate of entry exceeding that at which the contained crystalloids leak out of the cells. The eventual bursting of the cells presumably results from their inability to reach both osmotic and ionic equilibrium, because of their weak cell-walls and the retention of osmotically active substances inside the cell, which would still make the contents hypertonic even if the concentrations of potassium nitrate could become equal on the two sides of the cell-wall.

The transference of the swollen cells to sea-water is not followed by a return to their normal volume or by a crenation of the cells, as would be expected if the K^+ and NO_3^- ions were to leak out along the now reversed concentration gradient. Instead, both choanocytes and dermal cells produce several (usually not more than 5) blunt, cylindrical processes after about 7 minutes (fig. 1, B). These often contain the nucleus of the cell and sway about slowly. In one case the time for a complete to and fro swing was 60 seconds. After about three-quarters of an hour (23° C) the processes generally become free from the remainder of the cell by thinning and parting at the junction, but they do not appear to possess powers of locomotion.

The processes are not always produced when the tubes are transferred to sea-water, and much depends on the temperature of the potassium nitrate solution and on the time spent in this solution. Thus in squashed preparations (i.e. tubes transferred to sea-water for 30 seconds, then placed in a drop of sea-water on a slide and squashed under a coverslip to release the swollen cells), the processes were best seen when the tubes (*L. botryoides*) had been 40 to 60 minutes in the nitrate solution at 10·5° C; tubes only immersed for 30 minutes displayed the processes on few cells, while material transferred at 100 or more minutes showed very few processes. On the other hand, at 21°–22° C the processes were best seen if the tubes had been only 20 minutes in the potassium nitrate solution, few or none being seen after 40 minutes' immersion, though the dermal cells of tubes from the same specimen which had not been squashed, but left entire in the sea-water, produced well-defined processes after 45 minutes in the nitrate solution at the same temperature (22° C), but not after 70 minutes. A possible explanation for the latter difference would be that the dermal cells had survived longer than the choanocytes, for the latter are abundantly seen in the squashed preparations but are not

easily examined in the unsquashed tubes. However, this needs confirmation. The main conclusion is that the processes are best seen after the tubes have been left in the potassium nitrate solution for an optimal period depending on the temperature, between the limits of 10° C and 22° C.

A preliminary study has also indicated that the period of immersion in the potassium nitrate solution has some effect on the size and number of processes produced per cell: the number and (when only one per cell is produced) the length tend to decrease with increasing periods of immersion. It would appear that the cells must be swollen to a certain size before producing the processes, that the processes are best formed after an optimal period, and that too long in the nitrate solution causes the cells to lose their ability.

The formation of the processes can hence best be explained by a contraction of the stretched cell-wall, which softens at certain points so that the cell contents are forced out as tubular extensions. This is supported by the fact that immersion for 20 or more minutes in a solution containing 0·01 M potassium cyanide with 0·6 M potassium nitrate (22·5° C) prevents the formation of the processes after transference to sea-water, in both squashed and unsquashed material, whereas they are displayed by the control tubes after the same period in pure 0·6 M potassium nitrate. On the other hand, the flagella still beat in the solution containing the cyanide for as long as in the pure potassium nitrate solution, so that the former does not impair all the vital activities of the cells.

The cell processes are also formed when the tubes are transferred to isotonic calcium chloride instead of sea-water, though the former solution does not stiffen the mesogloea as much. Galtsoff (1925) noted that pure isotonic calcium chloride instantly killed the isolated cells of *Microciona prolifera*, but the flagella of the isolated collar-cells of *L. botryoides* remain beating for 5 to 10 minutes in this solution and the processes are produced. They even form in a solution of calcium chloride of $1\frac{1}{2}$ times isotonic strength, but not when the concentration is twice isotonic, or when a mixture (1:1) of calcium and sodium chlorides, each at twice isotonic strength, is used.

When the material is transferred to isotonic sodium chloride the processes appear, though the mesogloea hardly stiffens at all. The processes tend to be thinner, softer, and more numerous per cell, and in squashed preparations they are often crinkled and tend to wither rapidly at the junction with the rest of the cell. Numerous fine, filamentous processes may also be formed by the cells. The presence of calcium ions thus seems necessary for the stiffening of the cell-wall.

Before the conclusion of this section mention must be made of Huxley's (1921) 'finger cells' and 'clear cells'. The former appeared as rod-shaped cells attached in clusters to the inner surface of the pinacocytes of 'dermal blow-outs' produced by aggregates of previously dissociated cells of *Sycon coronatum*, while the latter were seen as isolated cells in older restitution spherules. The similarity between these and respectively the 'cells with processes' and the 'spherical cells' described above is obvious, and it seems possible that

some change in the ionic composition of the medium surrounding the cells of the restitution masses had taken place in an analogous way to that undergone by the tubes in the experiments described above. However, a few of the cells teased out of normal oscular tubes of *L. botryoides* have been observed to produce the long, blunt processes, and these are formed by isolated collar-cells of *Grantia compressa* (Dendy, 1914), so that the 'cell processes' may have a counterpart in the more normal animal. Dr. E. N. Willmer (personal communication) has also obtained similar processes (though not always) after dissociating specimens of *Sycon* by means of bolting silk some days after the material was collected.

Prior treatment with distilled water

Prolonged immersion of the oscular tubes in distilled water never results in cell or spicule dispersion, or in the sagging of the tubes, though some softening of the mesogloea takes place after several days. Thus after 10 days (20°–23° C) a tube of *L. botryoides* was still cylindrical and elastic, though corrosion had removed all but a few spicule remnants. This confirms that the reaction in potassium nitrate solution is not merely due to the removal of the ions of sea-water. Furthermore, the spicules are clearly not essential for the maintenance of the cylindrical shape of the tube, and this supports Maas's (1906) observation that larvae of *Sycandra raphanus*, grown in carbonate-free sea-water, can form a spongocoel and osculum without producing spicules at all; he adds that the ground substance takes over the supporting function of the spicules, being over-developed and hardened appropriately.

However, after immersion in distilled water for 20 minutes and then in the potassium nitrate solution, pieces of *L. complicata* undergo a rapid softening and sagging, but few spherical cells are to be seen. Most of the cells are disorganized by the preliminary treatment, and fixed preparations reveal burst nuclei and indistinct cell boundaries. Pieces and tubes of *L. botryoides*, however, survive the water immersion better, but after 1 hour (23° C) they show no spherical cells on being transferred to the nitrate solution. Only a mush of collar-cell remnants and clusters of cell contents in the jelly are to be seen as the tubes rapidly soften and the mesogloea swells. The difference between the species may perhaps be correlated with the occurrence of *L. botryoides* on weed exposed between the tide-marks, *L. complicata* being restricted to pools and to regions below low water.

The pieces and tubes flatten more quickly than the equivalent controls placed directly in the nitrate solution, the prior treatment with water having presumably rendered the surfaces more permeable by the disruption of the cells, and also possibly having modified the condition of the mesogloea by washing out some of the ions normally present. Immersion for several days in distilled water apparently alters the properties of the mesogloea further, for this does not swell to the same extent in the potassium nitrate solution, though the tubes immediately soften and become sticky. The mesogloea is re-stiffened on replacing the material in sea-water.

Preliminary treatment with even 95% alcohol (15 minutes), followed by washing with distilled water, likewise does not prevent the flattening of the tubes after 48 hours in potassium nitrate solution (13° C), though spherical cells and spicule spreading are not observed.

These experiments suggest that the potassium nitrate solution has a direct action on the mesogloea, for the cells are destroyed by the prior treatment with distilled water, and it seems an unnecessary complication to assume that the destruction of the cells has liberated an agent which causes the swelling of the mesogloea in the presence of potassium nitrate solution, but not of distilled water or sea-water, particularly in view of the known behaviour of hydrophilic colloids in ionic solutions. Reference has already been made to the action of calcium ions in stabilizing colloidal systems, but other ions also influence the state of dispersion of hydrophilic colloids (see p. 280). An alteration in the ionic composition of the medium may thus swing the balance between the sol and gel condition one way or the other, and hence, while doubtless the cells are able to soften the mesogloea in the normal sponge (in order to penetrate it or bring about a change in shape of the oscular tube), the behaviour of the oscular tubes in the potassium nitrate solution may be completely explained in terms of the direct action of the ions on the mesogloea and intercellular 'cement'.

This conclusion is supported by the behaviour of the oscular tubes in a solution containing 0·6 M potassium nitrate and 0·01 M potassium cyanide, for the tubes soften and collapse in the same time as the control tubes immersed in a pure solution of 0·65 M potassium nitrate, and yet the cells are impaired in the cyanide-containing solution because they are unable to produce cell-processes when transferred to sea-water.

Further support is derived from the behaviour of the tubes in some other isotonic salt solutions and this will be dealt with in the next section.

The behaviour of the oscular tubes in other isotonic salt solutions

The effects of immersing excised tubes of *L. botryoides* in some other isotonic salt solutions maintained usually at 15° C will now be briefly described. Several experiments have been performed, concerning either the direct comparison of the actions of all or some of the solutions on equivalent oscular tubes (usually in pairs), or the more careful study of the behaviour of the tubes in a particular solution.

Isotonic potassium chloride has the same action as potassium nitrate solution, but the rate of the reaction is much slower (see below). The tubes contract initially and then collapse with the swelling of the mesogloea and the dispersion of the spicules and swollen dermal cells. Much the same is found with lithium chloride, though the reaction is very slow and the mesogloea and cells do not swell so noticeably. With the sodium chloride solution, however, the tubes seem healthy for a long period, the pores remaining widely open, but eventually the mesogloea swells and the collar-cells (which do not swell) aggregate together to form a thin, central strand, or a series of clusters, of brown cells within the spicule 'lattice'. Apart from the fate of the pinacocytes,

which apparently fall away as in the former solutions, this result seems superficially similar to the 'involution' observed by Maas (1910) when using calcium-free sea-water, but this is perhaps to be expected since sea-water contains 70% isotonic sodium chloride. On the other hand, Galtsoff (1925) found that pure isotonic sodium chloride inhibited the amoeboid movement of isolated cells of *Microciona prolifera*, but this is not inconsistent, because the solutions used with the whole oscular tubes cannot be regarded as pure since calcium and magnesium ions will be supplied locally by the corrosion of the spicules.

In magnesium chloride solution the mesogloea does not swell much and the collar-cells remain in close contact with the main spicule zone. These cells aggregate together to some extent, for gaps appear in the choanoderm. Isotonic calcium chloride also produces a slight swelling and softening of the mesogloea, but only after many hours. Finally, potassium sulphate solution appears to kill the tube rapidly; some rounding-off of the cells in the oscular rim has been seen, but otherwise there is nothing to record after the pores have closed other than the rapid corrosion of the spicules. The tubes remain cylindrical for several days at least, with the mesogloea unswollen. Transference to potassium nitrate solution after 7 days produces no swelling or dispersion of the mesogloea, which is thus altered by the sulphate solution.

When a comparison is made between the times at which tubes from the same specimen become completely plastic in the different solutions (as indicated by pushing the tubes with a needle), the ions are found to fall into three series of decreasing softening (and swelling) ability concerning the mesogloea thus:

K^+	>	Na^+	>	Li^+	(chlorides)
4		19–22		26	
NO_3^-	>	Cl^-	≫	SO_4^{--}	(K^+—salts)
½		4		55	
	Mg^{++}	>	Ca^{++}		(chlorides)
	11–22		48–55		

(The figures represent the time in hours for the onset of plasticity; mean temperature 15° C.)

The onset of flexibility of the tubes will depend on such factors as the rate of penetration of the ions, their ability to soften the mesogloea, whether the tube contracts initially (increasing the interlocking of the spicules), or whether the cells secrete a softening enzyme, as well as on the state of the mesogloea. However, tubes treated for 3 hours with distilled water to destroy the cells have been found to give three similar series as regards the times at which a loss of elasticity was first detected, so that it is reasonable to assume that the onset of plasticity in the whole tubes is dependent on the direct action of the ions on the mesogloea. The three ionic series are the same as have been found for the dispersion of many hydrophilic organic colloids (Höber, 1950, p. 299).

These series may be explained as follows. Colloids tend to disperse in water when their constituent particles bear a net electric charge, since the particles then mutually repel each other. As the mesogloea does not disperse in distilled water, but does so in aqueous salt solutions, the gel is probably

tabilized by electrostatic linkages between oppositely-charged polar groups n adjacent particles. These linkages may be broken in salt solutions by the ombination of the fixed polar groups with the free anions and cations pro- ided by the dissociation of the salt; if the particles have a greater affinity for he cations than for the anions, say, they will acquire a residual positive charge nd the colloid will disperse. Since, however, the water molecule is electrically olarizable, the salt ions will be surrounded in aqueous solution by an en- elope of water, which will impede the tendency for combination to occur etween the fixed polar group and the free ion; for combination to take place, he energy of hydration must be overcome and water displaced from the urface of the ion.

The monovalent alkali cations when arranged in order of increasing atomic adius form the series:

$$Li < Na < K$$

The electric charge of each of these ions is the same, but the field-strength is reater at the surface of the smaller ions, which will therefore be surrounded y a thicker aqueous envelope in dilute solutions. In consequence the smallest ation, being the most hydrated, will have the greatest difficulty in combining vith the appropriate polar group on the colloidal particles, and hence the east tendency to disperse the colloid. This explanation, however, assumes that he polar groups are less polarizable than is the water molecule (for example, ulphate- or carboxyl-groups). In the case of polar groups which are more olarizable than the water molecule (for example, the phosphate-radicle), the ffinity between cations and the fixed polar groups will be greater than that etween the cations and water molecules, and lithium ions will therefore dis- erse the colloid more readily than will the other members of the monovalent lkali cation series at the same concentration (Teunissen, 1938).

The divalent alkali cations (calcium and magnesium), as mentioned earlier, an stabilize a colloidal gel by linking together fixed anions on adjacent col- oidal particles. The tendency for such linkages to occur will again be im- eded by the thickness of the water envelope, and the magnesium ion, being he smaller, will be the less effective stabilizer because of its thicker water film.

The free anions also form a series of decreasing dispersive power on account f their different polarizability (depending on the valency and ionic radius) nd their different degrees of hydration. By combination with polar groups on he colloidal particles they too can modify the residual charge on the particles, nd thereby influence the dispersion of the colloid.

Thus the behaviour of the oscular tubes in these other salt solutions is onsistent with the view that the ions are acting directly upon the mesogloea nd that this consists of an organic hydrophilic colloid similar to chondroitin ulphate, which possesses sulphate- and carboxyl-groups and which, com- ined with calcium, constitutes 40% of the dry weight of cartilage (Winter, 932).

Comparing the effects of the solutions in other respects, the healthy tubes ontract to some extent soon after being placed in each solution except the

potassium sulphate, while the pores close in a few minutes in all the solutions but the lithium and sodium chloride; in the former they have remained open for three-quarters of an hour, while in the latter they are widely open for about 3 hours (15° C). The spicules corrode in all the solutions, potassium sulphate being particularly rapid in this respect and the calcium chloride very slow, as would be expected. In all the solutions the corrosion affects the surfaces of the spicules that are transverse to the optic axis, as with potassium nitrate solution (Jones, 1955*a*). The cells swell in the potassium salts (except the sulphate), but do not swell so noticeably in the sodium, magnesium, calcium, and lithium chlorides, though this needs confirmation. Finally, as with potassium nitrate, flexibility tends to appear first at the oscular ends of the tubes. The reason for this is uncertain and may be due to several factors (for example, the permeability of the epithelia, the amount and firmness of the mesogloea, and the spicule size and concentration).

When combinations of isotonic chlorides in equal proportions were employed, the following series of increasing times (hours) for the onset of inelasticity of the tubes were obtained (one experiment, 15° C):

KCl	KCl+NaCl	NaCl
4½	18½	> 18½

KCl	KCl+$CaCl_2$	$CaCl_2$
4½	47	67

KCl+NaCl	KCl+$CaCl_2$	KCl+NaCl+$CaCl_2$	KCl+NaCl+$CaCl_2$+$MgCl_2$
18½	47	> 67 (flattening)	> 67 (firm)

These results conform with those of Gray (1920) concerning the gills of *Mytilus edulis*, and they are consistent with the above explanation of the influence of the cations on the stability of organic colloidal gels. Thus the K^+ ion in particular tends to disperse, whereas Ca^{++} tends to stabilize the gels so that the relative proportions of these ions in the isotonic mixtures will determine the stability of the mesogloea and the rate of dispersion, when this is favoured.

The action of acidified sea-water on oscular tubes

Solutions of acidified sea-water were made up by adding N/40, N/30, or N/20 hydrochloric acid to sea-water in the ratio 1 : 9 ml. These solutions will be designated N/40 a.s.w., N/30 a.s.w., and N/20 a.s.w. respectively in the following account. The pH of all three solutions was between 4·6 and 5·2, as shown by indicators. Forty millilitres of solution were used for each experiment.

The spicules of tubes and pieces of *L. complicata* were completely corroded away after 3, 6, and about 18 hours respectively in the N/20, N/30, and N/40 a.s.w. (19° C). After the calcite had disappeared, the tubes remained cylindrical and partly contracted, elastic, with closed pores, but without a swollen mesogloea; the collar-cells were rounded and the flagella inactive. Tubes of *L. botryoides*, on the other hand, survive the treatment for a longer period, another instance perhaps of the hardier nature of this species. Thus in N/20 a.s.w. the spicules have not dissolved after 18 hours (16° C), while with the N/30 or N/40 a.s.w. they are hardly corroded after 60 hours and the pores

main open and the flagella active for at least a day. The tubes undergo the st steps in the process of regeneration in the last two solutions, for the ithelia within 24 hours grow over their cut bases, and in the N/40 a.s.w. w spicules may even form. However, the material is not normal, for listers' are often produced on the inside of the wall of the tube by a local cretion or swelling of the mesogloea, while dermal 'blow-outs' of the type scribed by Huxley (1921) are common on the new wall covering the basal d of the tube. These 'blow-outs' are rapidly deflated by prodding them with bristle, and contain a watery fluid between the two cell-layers, while a few lls stretch across from layer to layer. Maas (1910) has also observed the velopment of 'blisters' on specimens of *L. lieberkühnii* which were left for veral weeks in filtered sea-water, but he attributed their development to arvation.

Transference of the tubes to sea-water, distilled water, or even 0·65 M otassium nitrate after all the spicules have dissolved fails to produce any preciable change in the material, but if the transference to the nitrate lution is made before the spicules have completely corroded, the usual welling and collapse of the tubes of both species takes place. The spicules ence seem to buffer the mesogloea against the fall in pH.

Thus prolonged immersion in N/20 acidified sea-water permanently ardens the mesogloea. On the other hand, it has been found that N/20 kaline sea-water softens the tubes.

Discussion

The observations recorded above are consistent with the view that the esogloea of the species studied consists of a colloidal gel, which firmly mbeds the spicules and which is capable of supporting the oscular tube after ne spicules have been dissolved. The mesogloea thus has a function similar o that of cartilage, and the spicules, while increasing the rigidity of the tube, re probably of equal importance as protective elements, since their sharp oints doubtless tend to make the sponge unpalatable for many animals. However, fish in captivity certainly eat the oscular tubes of even *L. complicata*. The spicules may also buffer the mesogloea against a fall in pH which would lter its properties as mentioned in the preceding section, while the slender monaxons constitute a system of props which keeps detritus away from the urface.

The presence of a layer of more easily dispersed jelly between the main picule zone and the choanoderm in *L. complicata* and *L. variabilis*, together vith the fact that the basal rays of the multi-rayed spicules tend to separate venly from the choanoderm as the tube grows (Jones, 1954*b*), indicates that he choanoderm is probably the source of the mesogloea. One would expect he collar-cells to be responsible for the secretion, since they are the feeding lements, though the amoeboid cells in contact with them, which are derived rom collar-cells (Duboscq and Tuzet, 1939), may manufacture the ground

substance. The secretion would appear to stiffen into the firmer gel as it passes to the outer spicule zone with the growth of the tube. There is no doubt that the mesogloea between the spicules progressively increases in extent, since photographic records confirming this have been obtained (Jones, 1952) and will be published in due course. The secretion of the mesogloea probably provides the pressure at the oscular rim needed for the longitudinal growth of the tube.

The evidence that the mesogloea is secreted by the choanoderm or associated amoebocytes is supported by the observation of Herlant-Meewis (1948*a*) that the amoebocytes in the deeper layers of the mesogloea of *Clathrina coriacea* contain refringent inclusions with the same staining properties as the hyaline collagen. Maas (1910) furthermore noted that a conspicuous increase in the structureless jelly occurred in between the choanocytes and the spicule layer when specimens of *Sycandra raphanus* and *L. lieberkühnii* were subjected to hunger, while Huxley (1911) observed the presence of organic matter within choanocyte 'blow-outs' obtained after the aggregation of dissociated collar-cells of *Sycon coronatum*, though he (1921) also noticed a gelatinous membrane around the older restitution spherules, which seemed of dermal origin. It is thus probable that the production of slime is a general property of both types of cell.

However, the evidence favours the collar-cells or amoebocytes derived therefrom as the source of the mesogloea, and not the dermal cells as Minchin (1900, p. 51) thought probable. This is important in regard to the hypothesis put forward briefly by myself (1954*a*) to explain the orientation of the spicules and their optic axes, for if the formative cell-sextets are to be oriented and tilted by the shear of the mesogloea past them, at least the inner layer of the mesogloea must consist of a fairly viscous fluid.

It seems unlikely that the firm mesogloea can function as a lymph in the transport of nutritive and waste substances as Loisel (1898) believed, though ions and small molecules no doubt diffuse through it. The cells, however, may derive nourishment by digesting its substance, as Herlant-Meewis (1948*b*) has observed in the developing gemmules of *Suberites domuncula*. The stiffness of the mesogloea would also preclude the possibility that it is contractile, which Loisel (1898) suggested after observing the movement and subsequent fusion of vacuoles in the mesogloea of species of *Reniera*. However, it may contract during the process of coagulation, while its surface tension when it is plastic may bring about a change in shape of the tube. For example, the oscular tubes of *L. variabilis* can become transformed into a series of bulges which round up into separate spheres or constrict off laterally to form diverticula. A similar production of diverticula in *L. botryoides* (*Ascandra variabilis* Haeckel) was noticed by Vasseur (1879), who decided that the sponge could propagate itself asexually by such means. The process is very similar to the formation of unduloids and diverticula by medullated nerve fibres from the vertebrate spinal cord when placed in hypertonic Ringer solution, and it is believed to depend on the surface tension of the myelin of the sheath (Young

45). Thus the surface tension of the mesogloea of the oscular tube, which is alogous to the myelin of the nerve-fibre, may be a factor in the determina-n of the shape of the tube when it is plastic. Schulze (1885), however, lieved that the mesogloea was not contractile.

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An Integration Method for the Interference Microscope

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SUMMARY

A method is described of measuring the integrated phase retardation of an object with a Baker interference microscope. With certain limitations, the total dry mass of the object can be derived from this figure. This method of 'weighing' objects is of particular value in measuring the growth of single cells, since it is very sensitive and can be used on a living cell without damaging it.

The method consists of restricting the field round the object with an aperture of known area and then measuring the total retardation within this field due to the presence of the object. The compensator is adjusted until there is an equal light intensity in the field with and without the presence of a quarter-wave plate. A similar adjustment is made with the object moved out of the field. The difference between the two compensator settings gives the integrated area retardation due to the object. The quarter-wave plate is motor-driven, and the light intensity is measured by a photomultiplier and presented on a cathode ray tube. The instrument is sensitive to a change of about 5×10^{-14} g in the dry weight of a biological object.

For an object of known specific refractive increment and mounted in water, the total dry mass can be derived from the retardation without the dimensions of the object being known. There is, however, a severe limitation with this method of integration—that the maximum retardation of the object must not exceed about 45° or $\lambda/8$. This can be shown by theory, and has also been confirmed by model experiments with small albuloidin spheres. Most biological objects have larger retardations in water, so it is necessary to mount them in media of higher refractive index. In this case, the volume of the object must also be known for the dry mass calculation. Although this limits the use of the method, it is important in measuring the growth of a cell to know the volume as well as the dry mass.

An example is shown of the application of this method in measuring the growth of a fission yeast, *Schizosaccharomyces pombe*. Curves are given showing the changes in dry mass, volume, and concentration of a single growing and dividing cell of this yeast.

INTRODUCTION

If a uniform object is examined with an interference microscope, it is possible to measure the retardation of a beam of light passing through the object. This retardation is a product of the thickness of the object and the refractive index difference between the object and the medium in which it is placed. Stated in this way, the measured retardation is only of limited use because in many cases both the thickness of a biological object and its refractive index are unknown quantities. Recently, however, two important facts about interference microscopy have been pointed out (Davies, Wilkins,

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Chayen, and La Cour, 1954; Barer and Joseph, 1954). First, that the dry ma
per unit area of an object in water is proportional to the retardation divide
by the specific refractive increment *irrespective of the thickness of the objec*
Secondly, that the specific refractive increment (α) for most cells can b
assumed to be approximately constant ($\chi = 100\alpha = 0{\cdot}18$). This means th
the total dry mass of a cell of uniform thickness and known area can b
calculated directly from the retardation (to an accuracy of about $\pm 10\%$
without knowing either its thickness or its detailed composition.

This result is very valuable, especially for sections in which the objects a
of uniform thickness. However, if the method is to be extended to living cell
the difficulty at once arises that most living cells are not of uniform thickne
and as a result the retardation is not the same over the area of the cell. Wh
is required in this case is a way of measuring the integrated area retardatio
of the cell, and the purpose of this paper is to describe one method of doi
this. The point of such a method is that it can give the total dry mass of
single cell. This is a very small quantity (roughly 10^{-10} g) and could hardl
be measured by any other existing technique except perhaps a very sensitiv
cartesian diver balance. An optical method of 'weighing' cells also has th
great advantage that it can be used on living cells without damaging them. I
is obvious that the technique may prove to be of considerable value, especiall
in the study of cell growth.

A preliminary account of this work has already been published (Passan
Mitchison, and Swann, 1955).

Method and Apparatus

The method we have adopted with the Baker interference microscope is t
place the object in an area of the field restricted by an aperture. The apertur
is trapezoid and can be altered both in shape and in area. We have called it th
'box'. The total retardation within the box is measured, first with the objec
enclosed within the box and then with the object moved out of the box. Th
difference between these readings is assumed to be the integrated area re
tardation of the cell ($= \sum_n r_n A_n$, where r is the retardation of a part of th
cell of area A) divided by the area of the box. This assumption, however, i
only valid if the maximum retardation in the object is less than about 45
(p. 291). In order to convert the measured retardation into the dry mass of a
object mounted in water, it is only necessary to know the area of the box an
the refractive increment of the object. It is not necessary to know any of th
dimensions of the object.

The crux of the method is the measurement of the total retardation within
the box, and the simplest way of explaining this is to describe how it evolved
The original idea, due to one of the authors (F. H. S.), was to make use of th
fact that there is a fairly uniform distribution of retardation over the back
lens of the objective (or any other conjugate plane) even though an irregula
distribution of retardation in the image plane would be caused by an objec

the box. The box was therefore placed in the image plane of a Ramsden eyepiece, and a $\times 20$ Becke lens was mounted above the eyepiece in order to examine the Ramsden circle. This Becke lens gave an image equivalent to the back lens of the objective when examining the object within the box. The retardation was nearly uniform over this image, and the next problem was to measure it. The simplest method of measuring a retardation is to set the light intensity to a minimum by adding an equal and opposite retardation from the compensator. This is not, however, very accurate and a better way with a uniform field is to use a matching method. Accordingly, a quarter-wave plate was put in at the level of the condenser iris so that it occupied half of the field seen in the Becke lens. The Sénarmont compensator (rotating eyepiece analyser and quarter-wave plate) was then adjusted until the two halves of the field were of equal intensity. The setting of the analyser for this matching condition was $22{\cdot}5^\circ$ from its position for minimum intensity, but this made no difference since it was only the difference between two successive readings that was important.

This arrangement was comparatively simple but had two disadvantages. First, the visual matching was not sufficiently accurate. It was difficult to get consistency of reading greater than $\pm 1^\circ$ rotation of the analyser ($= \pm 2^\circ$ retardation). Secondly, the image of the back lens of a high power objective was too small for easy measurement when viewed through the Becke lens. A compound microscope was tried in place of the Becke lens, but this arrangement proved very cumbersome.

The method that was finally adopted can be regarded as a modification of the one described above. The matching was done not on a split field but on consecutive presentations of a normal field followed by one with an extra quarter-wave retardation. A rotating disk presented alternately a blank hole and a retarding quarter-wave plate in the optical path, and the light intensities were compared by a photomultiplier mounted over the eyepiece. The output from the photomultiplier was amplified and presented on a cathode ray tube as two superimposed spikes, one representing the light intensity with the normal field, and the other the intensity with the quarter-wave plate. The analyser was then rotated until the heights of the two spikes was equal. This was the same match point as that obtained from the visual method, but the consistency of reading was 10 to 20 times better. Since a photometer automatically integrates the light intensity in the field, it was unnecessary to use the Becke lens to get a uniform field, and the photomultiplier was mounted directly over the eyepiece.

The present form of the apparatus is shown diagrammatically in fig. 1. The light source (*a*) is a 6 V 18 amp ribbon filament lamp run off accumulators. If the lamp is run with alternating current, there is an objectionable flutter in the traces on the cathode ray tube. The filters (*b*) consist of two 2-mm thicknesses of Chance ON20 heat-absorbing glass, and a yellow-green gelatine filter (Ilford No. 625). The peak transmission of the latter filter is about 33% at 5370 Å, which is very near the wavelength of the mercury green line

(5461 Å) for which the quarter-wave plates are selected. It is necessary check the transmissions of filter combinations in the infra-red and ultr violet, since photomultipliers may be sensitive in these regions. The inte ference microscope (*c*) is the normal Baker instrument with shearing objective but the analyser, the quarter-wave plate, and the upper part of the body hav been removed. The rotating disk (*d* and B) is mounted just above the micro scope. It has a quarter-wave plate (*l*) and a blank hole (*m*) at opposite sid near the periphery, and is driven at 1,425 r.p.m. by a small shaded-po induction motor. This speed is rather faster than is desirable, but it is difficu to get a small constant-speed motor which runs any slower. Small synchronou

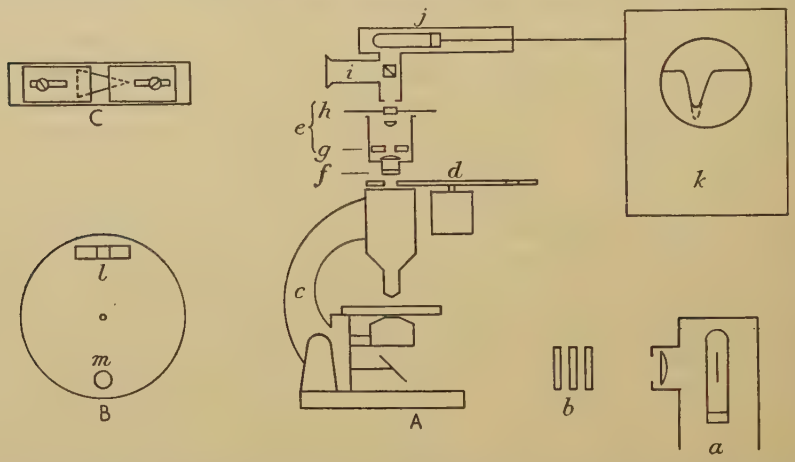

FIG. 1. A, integrating apparatus. B, top view of rotating disk. C, top view of 'box'. *a*, lamp *b*, filters; *c*, Baker interference microscope; *d*, rotating disk; *e*, eyepiece assembly; *f*, quarter wave plate; *g*, 'box'; *h*, rotating analyser; *i*, side-viewing eyepiece and prism; *j*, photomulti plier; *k*, cathode ray tube and amplifier; *l*, quarter-wave plate; *m*, blank hole.

motors are unsuitable, since they show speed fluctuations under load, an this produces a flutter on the final traces. The eyepiece assembly (*e*) is mounte above the disk. This consists of a Ramsden eyepiece with a fixed quarter-wav plate at the bottom (*f*), the box (*g*) between the lenses, and a rotating analyse (*h*) at the top. The box, which is shown in more detail in C, consists of triangular aperture (apex angle = 37°) and two sliding leaves. The size an shape of the trapezoid aperture can be altered by adjusting the position of th leaves. The rotating analyser is a small disk of cemented polaroid mounted i the centre of a Muirhead slow motion dial with friction drive. This dial has vernier reading to 0·1°, and a small reading light. Finally, there is a photo multiplier assembly above the eyepiece, consisting of a side viewing attach ment (*i*) with a movable prism, and a Photovolt photomultiplier in a light tight box (*j*). The output from the photomultiplier is amplified and presente on a cathode ray tube (*k*). The photomultiplier assembly, the eyepiec assembly, and the rotating disk are all mounted separately so that they can b swung away from the microscope axis.

Theory

In the method we have adopted, it is assumed that if there is an object of *uniform* retardation $r°$ occupying a relative area a of the box (a = area of object A/area of box A') and if a retardation of $x°$ has to be set in by the compensator to match the intensity of the fields with and without a quarter-wave plate, then:

$$x = Ar/A' = ar. \qquad \text{(a)}$$

This equation, however, is not accurate unless r is small, and the correct relationship for a uniform object is given by equation (3) in the Appendix:

$$\cot x = (1/a - 1)\operatorname{cosec} r + \cot r. \qquad \text{(b)}$$

The error involved in assuming equation (a) in the measurements can be shown by calculating x from a true value r with equation (b) and then getting a 'calculated' value of r by dividing this x by a. This calculated value is in fact the retardation that would be found experimentally with a uniform object, and it is slightly lower than the true retardation as long as the object fills less than half the area of the box. The results of a computation are shown in fig. 2. The continuous straight lines show true retardations of 30°, 45°, 60°, and 75°, and the curves in dashes show calculated retardations derived from these true retardations by the method described above. These are plotted against different ratios of object area to box area, but for the reason of convenience of scale the abscissa is not a but $1/a$ (= box area/object area). At 45°, the error (difference between true and calculated retardation) is 7·3% when $1/a = 10$ and 9·2% when $1/a = 50$. It therefore seems reasonable to take this value of 45° or $\lambda/8$ as an approximate upper limit for the retardation of an object which can be measured by this method. With higher retardations, the errors produced by assuming equation (a) increase rapidly, especially with large boxes. The curves also show that for retardations of 45° or less there is comparatively little change in the calculated retardation with different box sizes. This point is important when measuring the growth of cells because the relative area of box and object will change as a cell grows within a box of given size, and it is desirable that this change should not in itself affect the retardation. The curves in fig. 2 all cross the lines of true retardation when $1/a = 2$, which means that the calculated value is always correct when the object fills half the area of the box. It might in theory be possible to extend the range above retardations of 45° by using this fact, but there are two difficulties. First, as will be seen below, the limit for valid integration is also 45°, so it would only work for uniform objects. Secondly, there would be great practical difficulties in ensuring that the area of the box was always exactly twice the area of the object.

So far we have been considering the case of objects of uniform retardation. It is impossible to produce a simple solution similar to equation (b) for the

integrated total of an object of varying retardation. It is, however, possible to show by an argument which is given in the last section of the Appendix that the method used will give the integrated retardation of an object to within a maximum error of about 10% provided that the retardation of any part of the

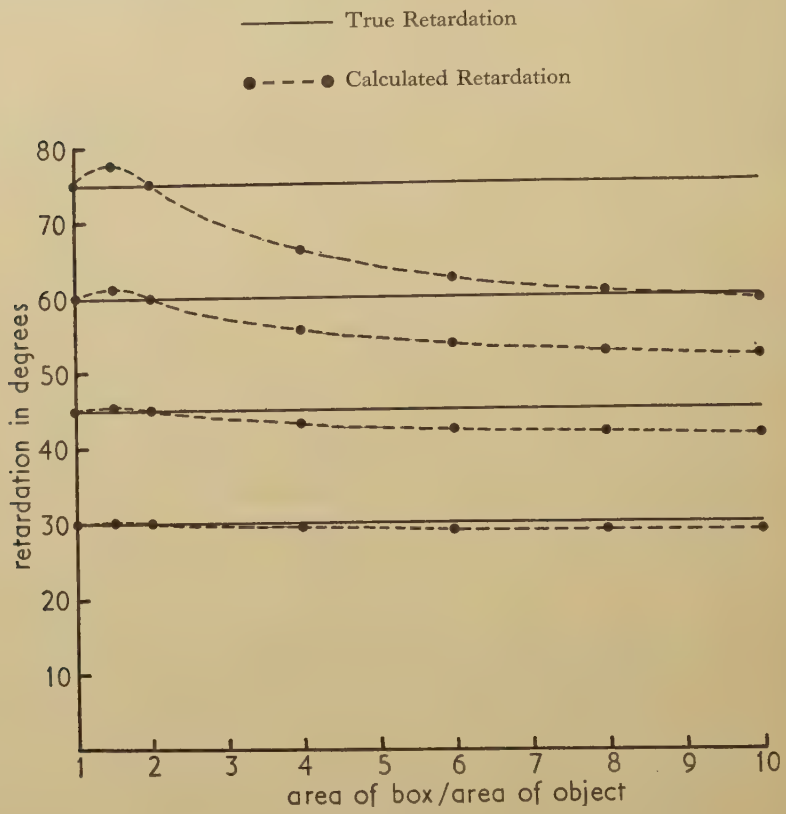

FIG. 2. Theoretical curves of object retardation against relative area of box.

object does not exceed 45°. This limit is the same as that given above for a uniform object.

Davies, Wilkins, Chayen, and La Cour (1954) have given the equations which can be used to calculate the dry mass of an object from the retardation. For an object in water:

$$m = \frac{\phi A}{\chi}, \qquad (c)$$

where m is the 'dry mass' or mass in grams of material other than water in the object; ϕ is the optical path difference of the object in centimetres ($\phi = r\lambda/360$, where λ is the wavelength of the light in centimetres); A is the area of the object in cm²; and χ is 100α, where α is the specific refractive increment of the object. For many biological objects, χ can be taken as 0·18.

For an object in a medium other than water:

$$m = \frac{\phi A}{\chi} - \frac{\chi'}{\chi} m', \tag{d}$$

where χ' is 100× the specific refractive increment of the medium; m' is the mass in grams of material other than water in a volume of the medium equal to that of the object ($= cV$, where c is the concentration of the medium in g/ml, and V is the volume of the object); and the other symbols are the same as those for equation (c).

In the method described in this paper, the object is measured within a box. In this case, for an object of uniform retardation:

$$\phi A = rA \times \lambda/360 = xA' \times \lambda/360, \tag{e}$$

where x is the total retardation within a box of area A'. This follows from equation (a) and is therefore valid only up to a maximum retardation of about 45°. Similarly, for an object of varying retardation and with an integrated area retardation $\sum_n r_n A_n$:

$$\sum_n \phi_n A_n = \sum_n r_n A_n \times \lambda/360 = xA' \times \lambda/360. \tag{f}$$

This follows from equation (4) in the Appendix and is also valid only up to a maximum retardation of about 45°.

If either of these two results is combined with equation (c), the dry weight of an object mounted in water can be determined without knowing the dimensions of the object. It is only necessary to measure the total retardation within the box and the area of the box. For an object in a medium other than water, however, it is also necessary to know its volume in order to calculate the last term of equation (d).

Measurements with Models

With a new method of this kind, it was desirable to test the validity of the theory by measurements on model objects whose integrated retardations could also be found by another method. The objects had to have a varying retardation in order to test the validity of the integration and they also had to have a simple geometric shape so that the integrated retardation could be calculated either from their refractive index or from a single measurement of retardation along one axis. One of the simplest shapes is a sphere. The integrated area retardation of a sphere is the cross-sectional area multiplied by 2/3 of the maximum retardation through the centre. This retardation is either measured directly or is taken as the product of the diameter times the refractive index difference between the sphere and its surrounding medium. (The mean optical thickness can be shown to be 2/3 of the diameter either by simply dividing the volume by the cross-sectional area—$(4/3\pi r^3)/(\pi r^2)$—or by formal integration.)

The model experiments were done with spheres, but there was considerable difficulty in getting spheres which satisfied two important criteria. First, the

spheres had to be accurately round. Although it was easy to see under a microscope whether or not a sphere was deformed or indented in the horizontal plane, it was almost impossible to detect this in the vertical plane. It was therefore necessary either to check that each sphere was round by rolling it with needles under a microscope (a method which could only be used with large solid spheres such as glass balls), or to use a batch of spheres where there were so few deformed ones that there was only a small chance that one of these was used for measurement. The second criterion was that the retardation of the sphere had to range between about 20° and 70°, since the theoretical limit for the integrating method was about 45°. This meant that either the diameter of the spheres or their refractive index difference from the medium had to be small. It was, however, difficult in practice to reduce the refractive index difference below a value which would allow for spheres any larger than about 20 μ in diameter.

Eventually, spheres of celloidin were selected as the most suitable models, but before this a number of other systems were tried. It is worth while to describe these briefly since they might be of use in providing models for other purposes.

Emulsion droplets. An emulsion was made of ethylene dichloride in a mixture of 85% glycerol, 15% water, and a trace of the commercial detergent 'Teepol'. The droplets of ethylene dichloride had nearly the same refractive index as the glycerol-water mixture and showed a wide range of size. They also had a density which was only slightly greater than the continuous phase, so they did not flatten appreciably under the action of gravity when they reached the bottom of the container. When viewed with a horizontal microscope, the majority of the droplets on the bottom were accurately spherical, but unfortunately there were an appreciable number which wetted the glass and flattened out. Since these flattened drops could not be distinguished from the others when viewed vertically, the emulsion could not be used for the integrating experiments.

Gelatine spheres. These were made by vigorous stirring at about 40°–50° C of a small quantity of liquid 20% gelatine (with a trace of Teepol) and a large quantity of hexane. The gelatine formed droplets, and if the mixture was allowed to cool while stirring was continued, these droplets set into solid spheres of gelatine gel. The spheres were brought down to water through ether and alcohol, hardened with formalin, and then placed in a glycerol-water mixture of nearly the same refractive index. The gelatine, however, did not emulsify easily, and as a result the spheres were several hundred microns in diameter, which was too large for the integration models. It might be possible to get smaller spheres with a better emulsifying agent.

Agar spheres. These were made by blowing hot liquid 1% agar through an atomizer with compressed air, and then catching the agar spheres (which solidified very quickly) in a basin of water. These spheres could be examined in water since they had a refractive index very near to that of water, but too many of them were deformed.

Glass spheres. Small glass spheres called 'ballotini' are made by the English Glass Co., Leicester. A fair proportion were accurately spherical and these could be selected by hand under a binocular microscope. The smallest, however, were about 80 μ in diameter, so the refractive index of the mounting medium would have had to be adjusted with great accuracy. Also, since the refractive index of the ballotini was variable, a separate medium would have to be made up for each sphere. This method was therefore rejected as being too long and inconvenient.

Celloidin spheres. The models finally adopted were celloidin spheres made by a simplified version of a method used in immunology (Kabat and Mayer, 1948). A 5% celloidin solution in alcohol-ether was layered over water. Small celloidin spheres of 2–8 μ diameter dropped down into the water from the interface, and these spheres were then washed, taken up through the alcohols, and finally suspended in immersion oil, terpineol, or glycerol according to the retardation which was required. Only a very small proportion of these spheres were deformed.

A thin layer of the suspension of celloidin spheres was mounted under a No. 0 coverslip and ringed with vaseline. Although the objectives of the interference microscope are corrected for a No. 1 coverslip, it was thought better to use a thinner cover in view of the high refractive index of the suspension. An isolated sphere was found and examined with the $\times$ 100 water-immersion shearing objective and a condenser N.A. of 0·3. The diameter of the sphere was measured with a micrometer eyepiece, and its maximum retardation through the centre was measured by five readings with a half-shade eyepiece (manufactured by C. Baker of Holborn Ltd. as an accessory to their microscope). The total retardation of the sphere was taken as 2/3 of this maximum retardation. It was not possible to calculate the retardation from the diameter of the sphere and the refractive indices of celloidin and of the mounting medium since measurements showed that there was considerable variation in the refractive indices of individual spheres. This difficulty also arose with the other model systems. The box was then set to a known area (measured by projection with a photographic enlarger) and inserted into the eyepiece. The sphere was centred within the box, and five readings taken of the analyser setting for equal height of the two spikes on the cathode ray tube. Another five readings were then taken with the sphere moved just out of the box. The retardation due to the sphere within the box was twice the difference between the averages of the two sets of readings. This retardation multiplied by the ratio of the box area over the cross-sectional area of the sphere gave the total retardation of the sphere. The procedure was then repeated with a number of other boxes of different area.

Results from these experiments can be plotted as curves of retardation against box size, which can be compared with the straight lines of the retardation from the half-shade eyepiece measurements. Fig. 3 shows the curves for 5 representative spheres out of the total of 14 which were measured. Similar curves were given by the other 9 spheres, but they have not been reproduced

in fig. 3 for reasons of clarity. It can be seen that with total retardations of about 50° and less, the retardation from the integration method is nearly the same as the retardation from the half-shade eyepiece measurements irrespective of the box size. This result appears to be somewhat more favourable than

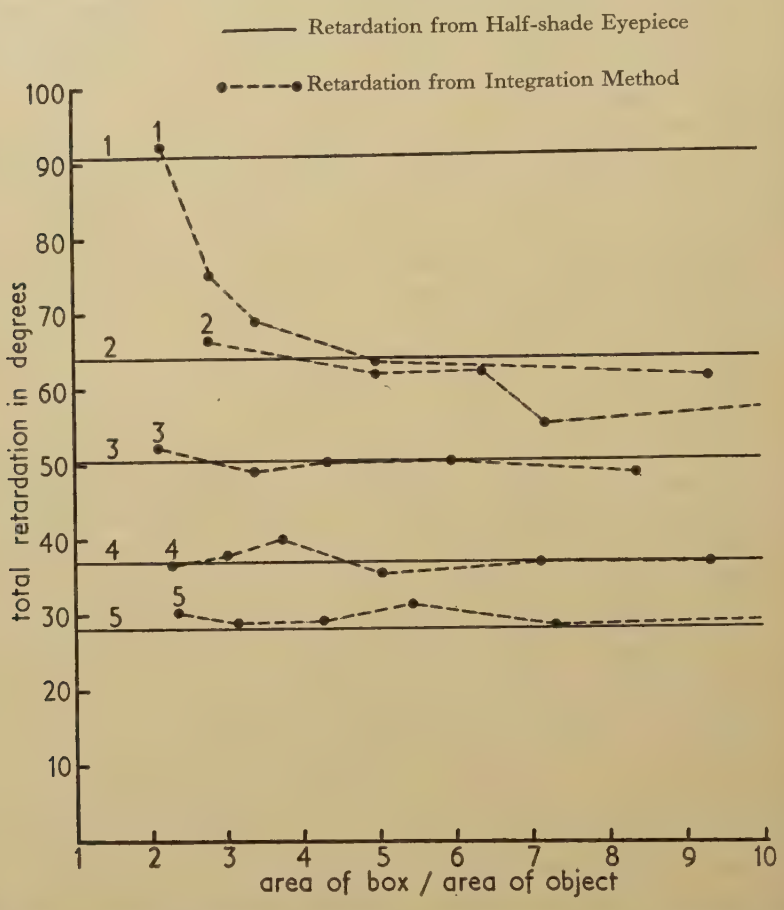

FIG. 3. Measurements on five celloidin spheres. Corresponding curves have the same number.

might be expected from theory since a sphere with a total retardation of 50° will have a maximum retardation of 75°. The simple theoretical treatment in the previous section set a rough upper limit of 45° both on the retardation of a uniform object which could be measured within a box and also on the maximum retardation in an irregular object. On the other hand, only a small portion of the sphere will have retardations near the maximum, and the error introduced in measuring this portion will have a comparatively small effect on the total measured retardation. The practical upper limit for the maximum retardation depends on the errors that can be tolerated, but it is unfortunately difficult to calculate the size of the error with this method even

when examining an object with a simple spherical shape. The safest criterion is perhaps to take the upper limit as 45°, but it should be borne in mind that there is likely to be only a small error in measuring a spherical or cylindrical object with a maximum retardation of 60°–70°.

The curves in fig. 3 have the same general shape as the theoretical curves in fig. 2, though it should be remembered that the experimental ones give the total result for an object of varying retardation while the theoretical ones give the result for an object of uniform retardation. Apart from this fact, there are three possible sources of error in the experimental curves which might account for their divergences from theory. First, the measurements with the half-shade eyepiece may not be strictly accurate. They depend on visual matching of the intensities on either side of a half-shade boundary placed over the middle of a small sphere in the microscope field. The error of this method may be as large as $\pm 4°$ of retardation. Secondly, the accuracy of the integrating method depends on the uniformity of the background. It is difficult to prevent slight variations in the background retardation due both to variations in slide and coverslip thickness and also to the presence of small specks of dirt. This may mean that the background retardation when the sphere is moved out of the box is not the same as that immediately surrounding the sphere. It may also produce an error when the box area is changed, since a large box may include a part of the background with a retardation different from that in a small box. The errors due to a non-uniform background probably account for the irregularities of the experimental curves. Thirdly, there is a possible source of error in internal and external reflection at the surface of the spheres, and in refraction of light rays passing through them. It is, however, unlikely that these effects made any significant difference to the measurements, since the refractive index difference between the spheres and the mounting medium was small (a maximum difference of 0·022 for spheres with total retardations less than 60°, and of 0·046 for spheres of higher retardations), and the illuminating cone from the condenser was narrow.

The accuracy of reading with the integrating method depends to a large extent on the size of the box. With a small box, only a small amount of light reaches the photomultiplier and it is difficult to equalize the spikes with accuracy because of electrical 'noise'. For five spheres in boxes of about $50\,\mu^2$ in area, the mean of the five standard deviations of each of the five analyser readings was 0·091°. For five spheres in larger boxes of about $130\,\mu^2$ in area, the mean was 0·042°.

It is reasonable to take the limit of sensitivity of the apparatus as about 5×10^{-14} g change in dry weight of a biological object. This implies a change in the analyser reading of 0·1° (the smallest angular change which can be read directly) with the object in a box of $30\,\mu^2$ in area (about the smallest size of box that is practicable at present). This limit of sensitivity is about one-quarter of the dry weight of a $1\,\mu$ cube of average cytoplasm. The apparatus could, however, be made more sensitive without any great difficulty. The electrical 'noise' which appears on the cathode ray tube could be reduced

either by using a stronger light source or a more sensitive photomultiplier and a finer reading device (e.g. a worm drive) could be fitted to the analyser.

Experiments with the celloidin spheres and with earlier model systems confirmed two results that would be expected by theory: that alterations in the shape of the box should not affect the reading provided the area was kept constant, and that the position of the object in the box should not affect the reading provided that it was contained within the box. In practice these changes caused small and irregular alterations in the readings, but these were almost certainly due to minor variations in the background.

The integrating system is comparatively insensitive to changes in focus. The object can be put out of focus to a considerable extent without altering the reading, provided the diffraction pattern of the object is contained within the box. This result is also what would be expected by theory, at any rate to a first approximation.

Measurements with Cells

It seems appropriate to conclude this paper with an example of how the integrating method can be applied to living cells. This example is only a preliminary result from a study of cell-growth which is being undertaken by one of the authors (J. M. M.) in collaboration with Professor M. M. Swann. A full description of this work will be published at a later date.

The major difficulty in applying the method to living cells is the stringent restriction that the minimum retardation should not exceed 45°. This retardation is given by a thickness of average cytoplasm of about 3 μ when the cell is in water or saline. Since most cells are either thicker than this or have local areas of higher retardation (e.g. the nucleus), it is necessary to reduce their retardation by mounting them in a medium of higher refractive index than water. As a result, the dry mass can only be calculated if the volume of the cell is also known (equation (d)). This restricts the use of this method for dry mass determination to those cells which have a regular shape whose volume can be calculated from the dimensions in the field of the microscope.

The cell that we have used is a fission yeast, *Schizosaccharomyces pombe* (N.C.Y.C. 132). This is a rod-shaped organism about 3 to 5 μ in diameter and 5 to 15 μ in length. It is a convenient cell for growth measurements for a number of reasons. First, it has a regular geometric shape—a cylinder with hemispherical ends—so its volume can be calculated from its length and diameter. Also, since it grows only in length, the increase in volume in a growing cell is directly proportional to the increase in length. Secondly, it divides into two daughter cells of equal size after the formation of a median cell-plate. This type of division has a greater resemblance to that of a normal plant or animal cell than the budding of other yeasts. Thirdly, like other micro-organisms it grows rapidly (up to one division every $2\frac{1}{2}$ hours at 30° C) in a sterile medium. Fourthly, unlike many bacteria, there is no reason to suppose that there is more than one nucleus in each cell or that nuclear and cytoplasmic division can get out of step.

The yeast cells were grown at 27·5° C in a weak gel with a nominal composition of 17·5% gelatin and 2% wort broth and an actual concentration of dry matter of 0·167 g/ml (for this medium $\chi' = 0{\cdot}186$). The gelatin had three functions. First, it provided a medium of sufficiently high refractive index to reduce the maximum retardation of the yeast cells to about 45°. Secondly, it kept the cells stationary and prevented them being moved out of the box either by Brownian movement or by slow drift. Thirdly, it enabled them to be suspended in the medium just below the coverslip. This allowed the use of the ×100 shearing objective, which has a very short working distance. The use of a weak gel appeared to have no adverse effects on the optical system. There were no signs of strain lines or distortions in the gel in the region round a growing cell.

Readings were taken (at $7\frac{1}{2}$-minute intervals) of the analyser setting with the yeast in the box and also of the setting for an adjacent empty background. Background readings were necessary each time since there were slow changes in the background retardation throughout the experiment which were probably due to very small changes in the tilt of the coverslip. Immediately after each retardation measurement, a photograph was taken of the yeast for calculation of the volume. The box size ($420\,\mu^2$) was left unchanged throughout the experiment. Although in theory alterations in the box size should produce no difference to the calculated dry mass, in practice they produce slight discontinuities in the growth curve due to minor background variations.

The most reliable measurement of growth so far obtained by this method is shown in fig. 4. This gives the changes in dry mass, in volume, and in concentration for a growing yeast-cell. The initial single cell divides into two daughter cells after 230 minutes, and the portions of the curves after this point refer to the total dry mass, volume, and percentage concentration of the two daughter cells. The volumes were calculated from the photographs, the dry weights from equation (d) assuming $\chi = 0{\cdot}18$, and the concentration from the dry mass divided by the volume (= 100× grams of material other than water per millilitre of cell volume). It is likely that the curves finish at about the point in interphase from which they started.

These curves are only intended to serve as an example of the method, and it is inappropriate at this point to discuss either their interpretation or their relevance to the many other published studies on cell-growth. In any case, this work is at an early stage, and it is impossible to say as yet how many of the minor details of these curves also occur with other cells. It is worth while, however, pointing out one thing about these curves which appears to be a general feature with all yeast-cells so far measured. For a period of about half an hour before cleavage, the volume stays constant but the dry weight increases. This is reflected in the rise of the concentration curve during the period. Whatever may be the meaning of this change from the point of view of cell behaviour, it does at least emphasize that for a single cell during one division cycle the growth in volume is not the same as the growth in dry weight, and that both these ought to be measured in an investigation of

individual cell-growth. It appears, therefore, that the volume measuremen serve a double purpose, even though they severely restrict the type of ce which can be used. Not only are they necessary for the dry weight dete minations but they also provide important information in themselves.

It is worth adding that although the main use of this integrating method

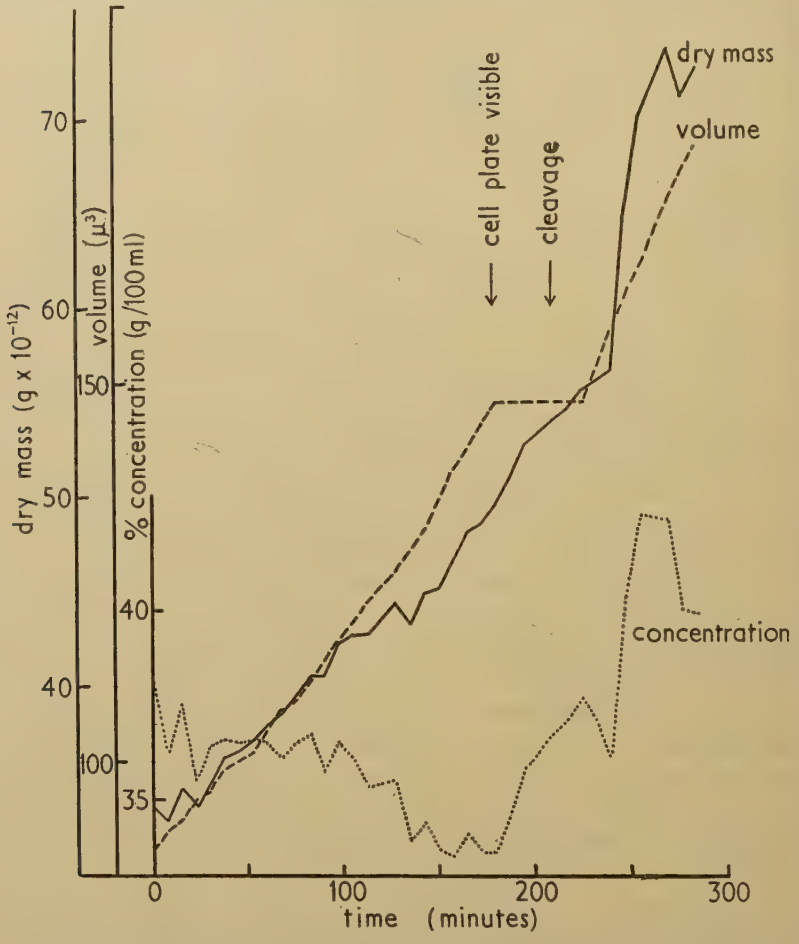

FIG. 4. Growth of *Schizosaccharomyces pombe*. One cell initially, which then divides to forr two daughter cells.

to provide a measure of the dry weight of a cell, there is also another possibl way of using it. If a cell can be shown to have a reasonably uniform refractiv index, this can be measured independently by the immersion technique c Barer and Joseph (1954). Provided this is known, the integrated retardatio can be used to calculate the volume of the cell, rather than its dry weight This might be of value in the case of a cell of irregular shape (e.g. *Amoeba* where it would be difficult to determine the volume by any other method.

In conclusion, it should be pointed out that there are other method

hich can be used to get integrated readings from an interference microscope. Davies and Deeley (1956) have described a method which they have used with he Dyson interference microscope. The object is placed in a field restricted y an aperture and the total light intensity due to the object is compared with hat from an external source of variable and calibrated brightness. A split eam system eliminates the errors which might otherwise be caused by uctuations of the light sources. One advantage of their method over that escribed in this paper is that the measurements are not affected by alterations n the relative area of object and aperture even with large retardations. The naximum permissible retardation, however, is of the same order in the two nethods.

Another method is to scan the object (or a photograph of the object), neasure the light intensity at each point, convert this to a retardation either y assuming the relation between intensity and retardation ($I\alpha \sin^2(r/2)$) or y using a calibration curve of intensity against known retardations from a ompensator, and then finally sum the retardations over the projected area of he object (Davies, Wilkins, Chayen, and La Cour, 1954; Caspersson, Carlson, nd Svensson, 1954). This method has the advantage of being able to measure arger retardations than is possible with the other methods which have been escribed, though there are certain inherent uncertainties unless measure-ents are made at two wavelengths (e.g. objects of retardations $\lambda/4$, $3\lambda/4$, $\lambda/4$, &c., all have the same intensity with light of a given wavelength). The ajor disadvantage is that it requires either a long time or relatively complex nd expensive equipment in order to carry out the scanning, measuring, and omputing.

APPENDIX

Derivation of equations for matching method

THE method consists of matching the intensity of two fields presented consecutively o the photomultiplier, one with and one without an extra retardation of $y°$. In the nstrument $y = 90°$, but the exact value does not matter for this argument. It is ssumed that the light intensity due to an object of retardation r is proportional to $\sin^2(r/2)$.

. No object in the field

For equal intensities of the two fields, let the retardation set in by the com-ensator be $z°$.

Then
$$\sin^2(z/2) = \sin^2(y/2-z/2),$$
$$z = y/2. \qquad (1)$$

. Object of uniform retardation in field

Let there be an object of uniform retardation $r°$ in the field, and let it occupy a elative area a of the field. The area of the rest of the field will then be $(1-a)$. For qual intensities of the field, let the retardation of the compensator decrease by $x°$ rom the initial retardation of $z°$ when there was no object. Then

$$\sin^2(z/2-x/2+r/2)+(1-a)\sin^2(z/2-x/2)$$
$$= a\sin^2(y/2-z/2+x/2-r/2)+(1-a)\sin^2(y/2-z/2+x/2).$$

From equation (1) $y/2 = z.$

Therefore

$$a\,[\sin^2(z/2-x/2+r/2)-\sin^2(z/2+x/2-r/2)]+ \\ +(1-a)\,[\sin^2(z/2-x/2)-\sin^2(z/2+x/2)] =$$

Using the relation $\sin^2 A-\sin^2 B = \sin(A+B)\sin(A-B)$,

$$a \sin z \sin(r-x)+(1-a)\sin z \sin(-x) = 0.$$

Dividing out by $\sin z$, and rearranging,

$$a \sin(x-r)+(1-a)\sin x = 0; \qquad (1$$

$$a \sin x \cos r-a \cos x \sin r+(1-a)\sin x = 0.$$

Dividing by $\sin x$,

$$a \cos r-a \cot x \sin r+1-a = 0.$$

Dividing by $a \sin r$, and rearranging,

$$\cot x = (1/a-1)\operatorname{cosec} r+\cot r. \qquad (1$$

3. *Object of varying retardation in field*

Let there be an object in the field with varying retardations $r_1, r_2, \ldots, r_n$, and eac retardation occupying relative areas $a_1, a_2, \ldots, a_n$, $(\sum_n a_n = a)$. This will give a equation similar to equation (2) above:

$$a_1 \sin(x-r_1)+a_2 \sin(x-r_2)+\ldots+a_n \sin(x-r_n)+(1-a)\sin x = 0.$$

This does not simplify easily unless it is assumed that

$$\sin(x-r) = (x-r).$$

In this case

$$x = a_1 r_1+a_2 r_2+\ldots+a_n r_n = \sum_n a_n r_n. \qquad (2$$

This is the answer required for a calculation of the total dry weight of the objec but it is only valid with the assumption $\sin(x-r) = (x-r)$. With a maximum erro of 10% this assumption is correct for values of $(x-r)$ of 45° or less, and therefor the integration is valid within this error for maximum retardations in the object o the order of 45° or less.

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The Structure of the Teeth of some Mammal-like Reptiles

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With two plates (figs. 1 and 2)

Summary

The principal changes in calcified tissues which have fossilized are the loss of organic material and the conversion of hydroxyapatite into fluorapatite. Since these two minerals are very similar and because, initially, mammalian enamel has a low organic content, its optical properties are hardly affected by fossilization. On the other hand, the loss of optically active collagen results in a modification of the characters of dentine.

The teeth of synapsid reptiles possess dentine similar to that of recent reptiles. Occasionally the dentine contained globuli, but unlike mammalian dentine, there appeared to be no 'spheritic' orientation of crystallites within the globuli. Certain dicynodont tusks consisted of dentine only and it is possible that enamel was missing even in the original condition. Other synapsid teeth possessed a thin, well-defined enamel layer made up of incremental lamellae but lacking true prisms. Nevertheless, cylindrical groups of crystallites exist throughout this enamel within which the orientation of the crystallite axes varies regularly. Between crossed nicols of a polarizing microscope this crystallite arrangement gives the enamel a prismatic appearance. However, the enamel is quite homogeneous, for these pseudo-prisms are not physically separated from each other. Furthermore, this regular prismatic appearance and an irregular *Säulengliederung*, such as occurs in crocodile enamel, may exist in the same tooth. There is, therefore, no evidence that the prismatic enamel characteristic of mammals existed in the pre-mammalian reptiles.

Introduction

CONSIDERABLE evidence has accumulated recently showing that the enamel-like tissue covering the teeth and scales of fish, as well as that covering the teeth of amphibians, is fundamentally different from mammalian tooth enamel (Levi, 1939, 1940; Kvam, 1946, 1950; Kerr, 1955; Poole, 1955). In these lower vertebrates the enamel-like material is of mesodermal origin, lacks prisms, and differs in other minor respects from the ectodermal, prismatic enamel of mammals. Reptilian enamel is more similar to that of mammals because it is ectodermal and its organic matrix is of a keratinous nature (Kvam, 1950). Moreover, hydroxyapatite crystallites are orientated in the same general direction in relation to the enamel surface, but as yet no prisms have been found in reptile enamel (Erler, 1935; Schmidt, 1948*a*, 1948*b*). In view of this, the problem of the origin of ectodermal prismatic enamel is of considerable interest and, for this reason, an examination of the teeth of pre-mammalian reptiles has been made.

The submicroscopic structure of mammalian teeth is now well known (Harders-Steinhauser, 1938; Thewlis, 1940) and the determination of the corresponding features of synapsid teeth has been a valuable way of comparing

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them with mammals. However, since all the teeth to be described a fossils of considerable age it was felt that before any structural interpret tions were made, careful consideration must be given to the possible effec of fossilization upon calcified tissues. To this end, a brief account of certa fossil mammalian teeth has also been included.

It has long been known that fossilizing bone accumulates fluorine, a fa which is of use in the estimation of the ages of fossil bone samples (Carnc 1893; Oakley and Hoskins, 1950). It has been suggested that the accumul tion is due to the replacement of $(OH)^-$ ions by F^- ions in the apatite lattic converting hydroxyapatite into fluorapatite (Oakley, 1948), and the results X-ray analyses of fossil specimens are in agreement with this (Poole, u published results). Both are negative, uniaxial minerals with very simil optical properties and, therefore, it would seem that the principal chang occurring in a calcified tissue undergoing fossilization are due to the loss organic material. Since enamel has a low organic content, few changes may b expected in it, whereas the properties of dentine, which initially contains considerable amount of optically active collagen, are likely to be modifie That the latter is so was shown by an observation of Schaffer (1891) th sections of fossil dentine reverse their sign of birefringence when transferre from xylene to Canada balsam. In doing so such sections behave in precise the same way as sections of recent dentine from which the collagen has bee removed by chemical means. Nevertheless, it is of interest in this connexio that it has been possible to identify collagen in the tusks of the late Siberia mammoth (*Elephas primigenius*) after a period of 10,000–15,000 years (Randa and co-workers, 1952). However, as pointed out by the same authors, th collagen structure would eventually break down at all temperatures abov zero over a sufficient length of time.

It is hoped that the account of fossil mammalian teeth given below wi help to amplify some of these points, and also serve to illustrate the fine stru ture of mammalian enamel and dentine generally.

Material and Methods

The mammalian teeth examined were from two Oligocene ruminant *Oreodon* and *Leptomeryx*. All the other teeth belonged to a range of mamma like reptiles collected in southern Africa and North America. Some of th specimens were not completely identified, but the following synapsid group were represented: Pelycosauria (*Dimetrodon*); Dicynodontia (*Lystrosaurus* an an unidentified genus); Gorgonopsia (unidentified); and Cynodonta (*Thr naxodon* and an unidentified genus).

Thin sections, both longitudinal and transverse, of all these teeth wer prepared and mounted in Canada balsam. Examination was made with norm and phase-contrast microscopes, and the optical properties of the variou tissues were determined by examination between the crossed nicols of polarizing microscope.

Fossil Mammalian Teeth

Ordinary microscopical examination revealed that the teeth of both *Oreodon* nd *Leptomeryx* possessed a typical orthodentine (see Orvig, 1951) similar in ll respects to the dentine of recent mammals. In polarized light the dentine f *Leptomeryx* had a uniform birefringence throughout, which was negative ith respect to the tooth surface and indicated that the negatively birefringent rystallites of apatite are arranged with their optic axes parallel with the surace of the tooth. A similar crystallite arrangement existed in *Oreodon* dentine, ut in addition a well-marked 'spheritic' orientation was superimposed upon . This is suggested by the occurrence of whole, partial, or distorted circles, s seen in fig. 1, A, each of which is marked by a polarization cross. Both types f orientation are to be found in recent mammalian dentine (Keil, 1939).

In both specimens the sign of birefringence reversed on transferring a ection from xylene to a medium of higher refractive index. In recent dentine he positive birefringence of the collagen overcompensates that of the negaive mineral; if these fibres are removed (e.g. by boiling in glycerol-potash olution), spaces are left which, when penetrated by a liquid of quite different efractive index from that of the mineral, set up a positive 'form' birefringence, gain overcompensating the mineral. However, when a liquid with a refracive index nearer to that of the mineral is used, the 'form' birefringence is emoved and only that of the mineral remains. This accounts for the reversal f birefringence when fossil dentine, or recent dentine from which the colagen has been removed, is transferred from xylene to Canada balsam.

As was anticipated, the properties of fossilized enamel proved to be very imilar to those of recent enamel. The prisms were perhaps a little less bvious, possibly because the loss of organic material from the interprismatic ubstance decreased the relief between it and the actual prism substance. Nevertheless, the characteristic cross-striation of the prisms was still apparent under all conditions and this is also seen very clearly in the enamel of ertain fossil rodents (Korvenkontio, 1934).

Between crossed nicols each prism exhibits a negative birefringence with espect to its length, so that the crystallites within must lie with their optic xes approximately parallel with the prism axis. By rotating a section it is ound that the extinction position of the prisms is different from that of the nterprismatic substance. Fig. 1, A shows an enamel layer close to an extinction position and producing, as a result, anomalous polarization effects. In nany places the prisms are extinct and appear dark, whilst the thinner interprismatic zones separating them are still light. Since the prism direction varies omewhat, certain prisms which have passed the extinction position may also e seen; these are light whereas the interprismatic substance is now dark. The same effect is true of human enamel, as illustrated in fig. 2, D and E. Thus, as in recent enamel (Thewlis, 1940), the crystallites in the interprismatic ubstance of fossil enamel are not parallel with those within the prisms. Gustafson (1945) distinguishes carefully between prism sheath and the very

thin interprismatic cementing material, concluding that it is the former, largely organic region, which is responsible for the different properties of th prisms and the material which separates them. Yet this difference persist even in fossilized enamel, so that if all organic material is presumed lost, th difference must be due to mineral whatever its exact location may be. In thi account, the term 'interprismatic substance' will be used loosely to describ all the material occurring between the prisms.

The zonation of the enamel seen in fig. 1, A is due to striae of Retziu running out gradually from the amelodentinal junction across the prisms an eventually reaching the enamel surface. The effect is caused by slight dis placement of the prisms during formation (Gustafson, 1945) and is we known in human enamel. As with recent enamel (fig. 2, D), the cross-striatio of the prisms is seen clearly in polarized light. In addition the activity of th innermost enamel is considerably greater than elsewhere, for in this regio the prisms are comparatively straight and parallel throughout the section; o moving outwards, considerable bending and twisting occurs and the effect of surface prisms are partially compensated by those of more deeply lyin prisms running in a slightly different direction. As a result of this there is reduction in the overall activity of the outer enamel.

This brief account is sufficient to demonstrate that all the importan features of mammalian enamel are retained after fossilization. The onl important change in fossil dentine is the loss of collagen, and the propertie of the dentine are still very much the same as those of recent dentine fro which collagen has been removed artificially. Therefore, the methods c examination outlined above should be a valuable guide to the presence o absence of prismatic enamel in fossil material of even greater antiquity tha that already dealt with.

Teeth of Mammal-like Reptiles

Some of the material examined was very well preserved; the cynodont teet were still socketed in jaws with the result that sections of the whole specime yielded information concerning all of the tooth tissues, including cementum and the bone of the jaws. The insertion of the teeth resembled that of mammals both primary and secondary cement layers being distinguished.

The orthodentine showed very constant properties throughout the rang of teeth studied. Dentinal tubules ran out from the pulp cavity towards th amelodentinal junction, and a thin, structureless layer occurred between th ends of the tubules and the beginning of the enamel. Longitudinal section had a negative birefringence with respect to the tooth surface, whereas transverse sections showed little or no activity between crossed nicols. I

Fig. 1 (plate). A, longitudinal section of a tooth of *Leptomeryx* arranged close to an extinc tion position between crossed nicols. The enamel prisms are crossed by striae of Retzius whil illuminated arcs and circles occur throughout the dentine.

B, longitudinal section of a cynodont tooth under the same conditions. The presence o alternating light and dark zones in the enamel produces a superficial resemblance to mammalia enamel. This pattern is somewhat obscured in certain parts of the enamel.

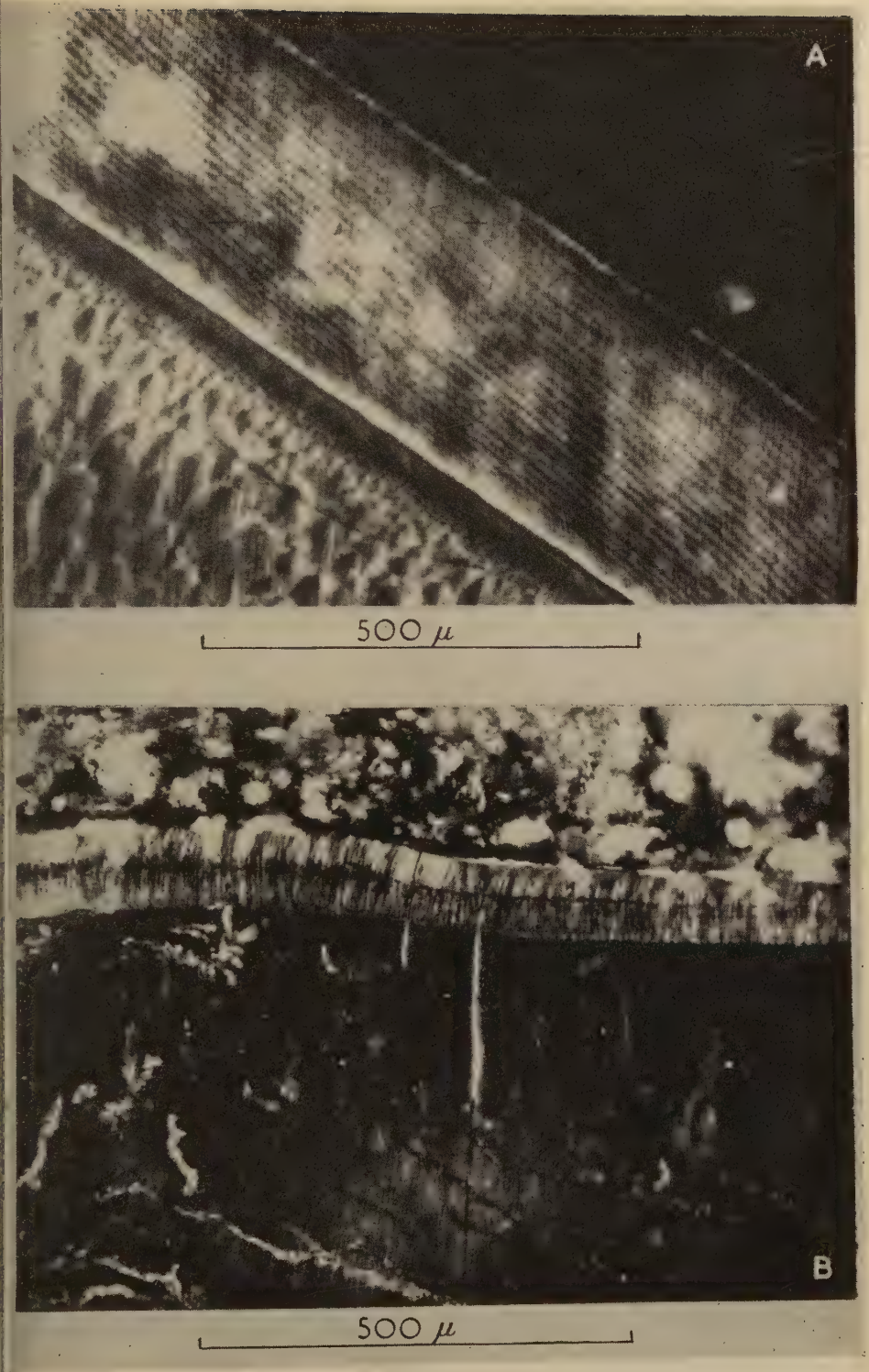

FIG. 1

D. F. G. POOLE

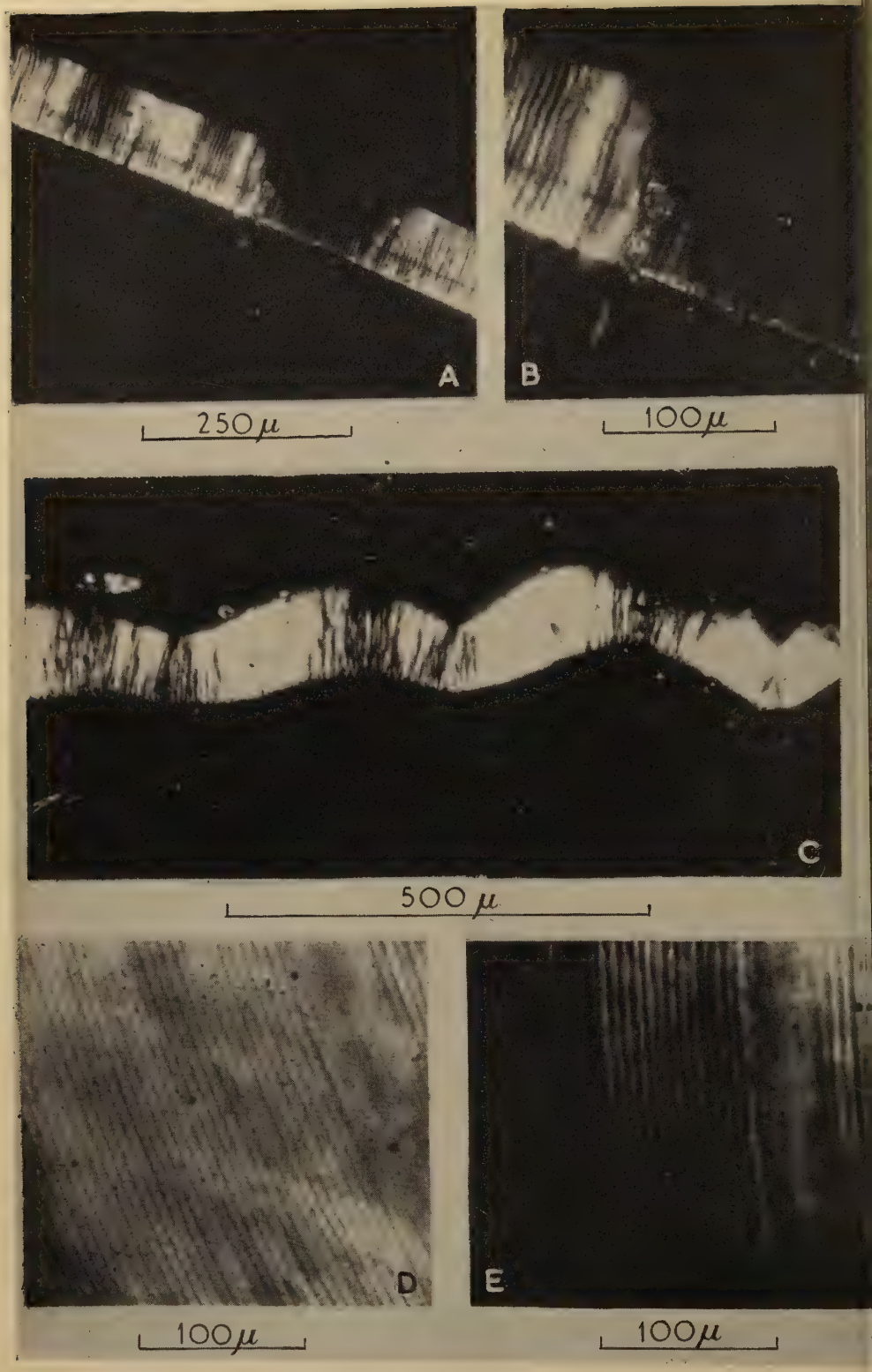

FIG. 2

D. F. G. POOLE

general, therefore, apatite crystallites are arranged with their optic axes parallel with the surface and the long axis of the tooth. Occasionally, patterns of arcades or circles were seen between crossed nicols, particularly in the dentine of *Dimetrodon*, but these differed from those of a similar shape and size in mammalian dentine in that polarization crosses were either weak or absent. Very small globuli, again lacking polarization crosses, may be seen in crocodile dentine where all the crystallites lie with their optic axes parallel with the direction of the collagen fibres. This suggests that the presence of globuli does not necessarily imply a 'spheritic' orientation of crystallites such as occurs in mammals, and that it is possible for crystallites within the spheres to be orientated in the same general direction as the collagen. This view is shared by Schmidt (1955).

The dicynodont tusks possessed no enamel; one of these was weathered on one side but the other side seemed to be quite intact, and, furthermore, the tusk of *Lystrosaurus* was still completely embedded in rock. The general characters strongly suggested that, had it ever existed, an enamel layer ought still to be present. Enamel may have been lacking even in the original condition, for the tusks of *Lystrosaurus* grew continuously from persistent pulps (Broom, 1932) and a parallel could exist with certain mammals where this is also true; e.g. elephant and *Babirusa*. In such cases the production of dentine is continuous, but the enamel organ ceases to function after eruption and only the original tip is covered by enamel.

The enamel covering the cynodont and gorgonopsid teeth was well defined but very thin, being no more than 0·1 mm in thickness on a tooth with a long diameter of 15 mm. With ordinary microscopic examination gorgonopsid enamel appeared almost structureless except for thin, faint lines parallel with the enamel surface breaking it up into lamellae. The same was true of cynodont enamel, but in this case many tubule-like spaces, lying at right angles to the amelodentinal junction, were present in the innermost region. Between crossed nicols this enamel again appears almost structureless when it is arranged in the position of maximum illumination and it has a positive birefringence with respect to its surface, indicating that the negative mineral crystallites lie, in general, with their optic axes perpendicular to the surface. However, if the gorgonopsid or cynodont enamel is now rotated towards

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Fig. 2 (plate). The enamel of various teeth as seen in polarized light; in each case the enamel is arranged near to an extinction position.

A, transverse section of gorgonopsid enamel with a well-marked 'prismatic' appearance and with very thin layer lines running parallel with the surface.

B, enlargement of a fragmented portion of the same gorgonopsid enamel showing that the characteristic pattern is still visible even in very thin sections.

C, transverse section of the enamel of *Dimetrodon*, again with layer lines and a simulated 'prismatic' appearance.

D, human enamel possessing illuminated prisms separated by thinner, dark, interprismatic regions.

E, the same human enamel rotated so that the prisms are extinguished. Since the crystallites within the prisms have a different orientation from those in the interprismatic substance, the latter now lights up.

extinction position a new pattern is produced, which is illustrated in figs. 1, B and 2, A and B. Here are seen alternating black and white lines perpendicular to the enamel surface, crossed by the lamellae, and bearing a superficial resemblance to mammalian prisms examined under the same conditions. The effect is clearer in gorgonopsid enamel than in cynodont, where it is obscured by the tubule-like spaces (fig. 1, B).

Despite the superficial resemblance, the effect described above differs in several important respects from mammalian enamel. For example, it only manifests itself under certain circumstances, whereas mammalian prisms are visible under all conditions. Fig. 2, D is a photograph of mammalian enamel arranged close to an extinction position between crossed nicols; the illuminated prisms are cross-striated and separated from each other by the much thinner interprismatic regions. The latter are never cross-striated and here they appear dark because the crystallites within them are extinguished. The same enamel, rotated so that the prisms have become extinguished, is shown in fig. 2, E; because of their different orientation the crystallites of the interprismatic material have now passed the extinction position and begin to light up. The important point is that the pattern of broader prisms separated by thinner zones of interprismatic substance is always the same. In contrast to this, the apparent prismatic appearance of synapsid enamel is more precise when the enamel is parallel with an extinction position than when the enamel is in any other position, although there is always a tendency for the black and white zones to grade into each other through shades of grey. On rotating a section from the extinction position, the black lines, which are in general thinner than the white for any position of the enamel, move gradually across areas originally illuminated. As the rotation is continued, all zones become less and less distinct until at 45° from extinction, the position of maximum illumination, little trace of the prismatic pattern remains. If the enamel is now returned to the extinction position and a sensitive tint (first order red) quartz plate is inserted at 45°, a very interesting colour pattern is produced. In very thin enamel there is a regular repetition of a blue-red-orange colour sequence, so that the prism appearance is produced by groups of crystallites whose orientation varies regularly about a normal to the enamel surface. All these points indicate that synapsid enamel is not broken up into units separated from each other by material with rather different properties, but is a continuous homogeneous layer of crystallites the orientation of which varies slightly but regularly. In other words, these are not true prisms.

Although true prisms may not exist in synapsid enamel, by focusing at different depths of a thick section it may be observed that individual groups of crystallites exist throughout the enamel. Furthermore, when a section parallel with the surface is viewed between crossed nicols, a mosaic of small circles, each with a diameter equal to the width of a 'prism', is observed. This means that each group of crystallites is in fact cylindrical in shape and runs from the amelodentinal junction to the enamel surface. As shown above, these cylinders are not physically separated from each other. The 'prisms'

could not be seen by ordinary phase-contrast microscopy, but when a source of plane-polarized light is substituted the effect is again noticeable as a series of alternating lighter and darker zones, that is to say, alternating areas of positive and negative phase.

To explain these various properties of synapsid enamel, the following structure is proposed. Fig. 3, A represents a section of enamel built up of a series of lamellae which undulate regularly. These undulations can actually be observed in a specimen, but since the groups of crystallites are cylindrical, any one lamella does not consist of elongated corrugations but of hemispherical elevations and depressions. Crystallites tend to lie with their optic axes at right angles to the lamellae, but, as shown in fig. 3, A, because of the

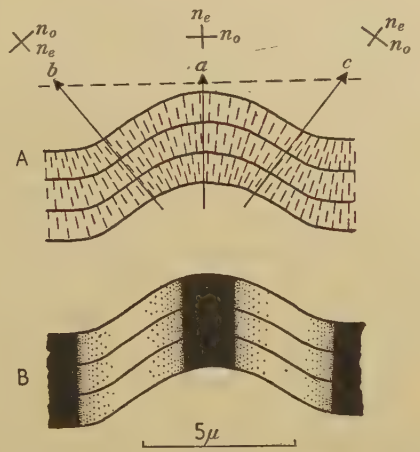

FIG. 3. Diagrams of a suggested structure of gorgonopsid enamel. A, the enamel is made up of lamellae containing crystallites. The crystallite and optic axes, which are coincident, are at right angles to the undulating lamellae so that there is a regular variation in crystallite direction, e.g. *a*, *b*, and *c*. The two vibration directions of the crystallites, with differing refractive indices n_e and n_o, are indicated by mutually perpendicular axes. B, the appearance produced in polarized light by such a crystallite arrangement when the enamel as a whole is parallel with an extinction position. Crystallites along direction *a* will appear dark, those along *b* and *c* will be illuminated. Light and dark zones tend to grade into each other.

undulations there will be a variation in the crystallite direction about the surface normal *a*. If the enamel as a whole is arranged parallel with an extinction position the condition shown in fig. 3, B is produced; all the crystallites with their optic axes parallel with direction *a* will be extinct and the area around them dark. On the other hand, crystallites parallel with directions *b* and *c* will be illuminated, but since the orientation changes only gradually, the light and dark zones tend to grade into each other. Should a quartz sensitive plate be placed with its positive axis parallel with direction *c*, the crystallites here will become orange-yellow because the mineral is negatively birefringent; crystallites along direction *a* are inactive and will appear red, whilst those parallel with *b* will become blue-green. Furthermore, if the enamel in fig. 3, A is rotated, the orientation of the different groups relative to the

extinction position becomes less regular and the prism-like pattern less and less distinct as the 45° position is approached.

An arrangement of mineral such as this also accounts for the prism-like effect produced with a phase-contrast microscope and plane-polarized light. In fig. 3, A the vibration directions of crystallites are represented by two mutually perpendicular axes and, since fluorapatite is a negatively birefringent, uniaxial mineral, the refractive index along the optic axis (n_e) is smaller than that at right angles to it (n_o). When plane-polarized light vibrates through the enamel parallel with the direction *c*, it will be subjected to the lesser refractive index (n_e) of crystallites lying in this direction but to the greater refractive index (n_o) of those with their optic axes parallel with direction *b*. With the phase-contrast microscope these areas of alternating refractive index will appear as zones of positive and negative phase. If the section is rotated so that the plane-polarized light is now vibrating parallel with direction *b*, a change of phase in each zone will result. The crystallite arrangement suggested in fig. 3, A does, therefore, account for all the observed properties of this enamel.

Finally, brief mention of pelycosaur enamel must be made. The teeth of *Dimetrodon* are compressed laterally, sections parallel with the long, transverse axis again showing a simulated prismatic appearance (fig. 2, C). However, sections along the shorter axis presented no distinct pattern, resembling the irregular *Säulengliederung* typical of crocodile enamel (Schmidt, 1948*a*). It is possible, therefore, for a variation of pattern to occur in the same tooth.

Discussion

As a result of the investigations carried out it seems that enamel consisting of individual, separate prisms did not occur on the teeth of mammal-like reptiles. Although, in some cases, the enamel is composed throughout of regular, cylindrical groups of crystallites, in others variation occurred from this condition to that of the more irregular *Säulengliederung* first described in crocodile enamel. In the enamel of certain placodonts the two conditions may also be found, and the *Säulengliederung* is to be seen in the enamel of certain other fossil parapsids (Schmidt, 1948*a*, 1948*b*). Recent investigations show that the same general properties are possessed by the enamel of pterosaurs (Schmidt, 1955), cotylosaurs, and recent Squamata (Poole, unpublished results), so that if the 'prismatic' effect is simply a more regular form of the *Säulengliederung*, there is a very constant enamel structure throughout a wide range of reptiles.

Unless true prismatic enamel is found in some other more direct, and possibly unknown, reptilian ancestor, it must be presumed that it originated with the early mammals. At this stage a number of specializations of mammalian teeth and feeding habits took place; for instance, there was the development of the habit of mastication, causing greater wear on the teeth and subjecting them to increased mechanical stresses and strains as well as to the actions of saliva and mouth-acids. In addition, there was a reduction in the total number

of teeth during the life of an animal, only a limited number of sets being formed. Even the advanced cynodonts differed from the mammals in that many of the teeth were replaced several times and the replacement of the incisors was alternate or 'ditischic' (Crompton, 1955). Thus the increased thickness of mammalian enamel might well be a response to new functions of the teeth, and it is worth noting that the massive teeth of placodonts, used for crushing, had an enamel layer much thicker than that covering the teeth of other reptiles. It is also possible that the prismatic properties of mammalian enamel confer greater mechanical advantages in localizing the effects of crushing forces which would be spread over a much larger area in a thin shell of material.

Nevertheless, there are many points of similarity between reptilian and mammalian enamels. The histological appearance of the developing teeth of a crocodile embryo is very similar to that of mammals, and in each case the orientation of mineral crystallites in relation to the enamel surface is the same. Each cylindrical group of crystallites in gorgonopsid enamel may well be the product of one ameloblast, since it is of the correct dimensions; if so, mammalian enamel could have arisen by the extended growth of the ameloblasts, each one eventually acting independently in forming a separate calcified rod surrounded by an organic sheath representing the remains of the original matrix.

Perhaps the whole problem has been oversimplified here, and, indeed, no consideration has been given to the 'tubular' enamel of marsupials and certain other mammals in the proposed evolutionary story. Since no recent accounts of this type of enamel seem to exist and earlier ones (e.g. Tomes, 1897; Mummery, 1914) resulted in differing views, the true affinities with the more usual type of mammalian enamel cannot be assessed. Nevertheless, evidence is in favour of the view that the reptilian enamel described above may be some sort of direct precursor of mammalian enamel. For this reason, an investigation is at present being made into the formation of crocodile enamel, which, since it is comparatively simple in structure, may possibly yield information on some of the fundamental properties of all ectodermal enamels.

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Volume 97. Part 2

CONTENTS

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